SCREENING FOR BIOGENIC AMINES PRODUCTION BY *LACTOBACILLUS* SPECIES AND DEVELOPMENT OF FUNCTIONAL FOOD, TEA CURD

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CERTIFICATE

This is to certify that the thesis entitled **"SCREENING FOR BIOGENIC AMINE PRODUCTION BY *LACTOBACILLUS* SPECIES AND DEVELOPMENT OF FUNCTIONAL FOOD, TEA CURD"** which is being submitted by **Miss. Banishree Sahoo,** Roll No. **411LS2125,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that the thesis entitled **"SCREENING FOR BIOGENIC AMINES PRODUCTION BY *LACTOBACILLUS* SPECIES AND DEVELOPMENT OF FUNCTIONAL FOOD, TEA CURD"**, that I submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a record of bonafied and original research work carried out by me under the guidance and supervision of Dr. R. Jayabalan, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other university or institution for the award of any degree or diploma.

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ABSTRACT

Biogenic amines (BA) are substances with one or more than one amine groups.BA is organic, basic nitrogenous compounds of low molecular weight. Biogenic amines (BA) are natural toxins. Removal of the α-carboxyl group from an amino acid leads to the corresponding biogenic amine. BA generally occurs in many different kinds of food, such as fishery products, cheese, wine, beer, dry sausages and other fermented foods like curd and idli batter. Functional food is a food, where new ingredients or more of existing ingredients have been added to a food and the new product has an additional function which relate to health-promotion or disease prevention. Potential of functional foods are to mitigate disease, promote health and reduce health care costs. Screening of non-biogenic amine producing LAB from OMFED and homemade curd was done using decarboxylase media. The bacteria having decarboxylase activity gave purple zone due to production of basic amines. Out of five strains only two were found to produce biogenic amines while the other three gave negative result. Those which did not produce biogenic amines are then utilized to prepare green and black tea curds. These curds contain health enhancing compounds such as catechins and flavonoids from tea and also the probiotic LAB strains helpful in many ways. All the three negative strains showed acid tolerance upto pH 3 while they could not survive in pH lower than that. Hence they can be successfully used in curd production as probiotic bacteria since they can tolerate the acidic pH of the upper part of GIT.

Key Words: Biogenic amines, Functional foods, Flavonoids, Catechins.

INTRODUCTION

Biogenic amines (BA) are biogenic substances with one or more than one amine groups.BA are organic, basic nitrogenous compounds of low molecular weight (Bodmer et al., 1999). Biogenic amines are natural toxins. Removal of the α-carboxyl group from an amino acid leads to the corresponding biogenic amine. These are known as biologically active molecules having aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine, histamine, tryptamine) structures. BA are organic basic compounds generallyoccurring in many different kinds of food, such as fishery products, cheese,wine beer dry sausages and other fermented food(Brink et al., 1990). It is also found in raw and fermented foods (Shalaby, 1996).Biogenic amines are alkaline in nature. During digestion, the pH of the human gastric environment can decrease to values below pH 2. The main bacteria which are responsible for BA production in fermented food matrices are the lactic acid bacteria(Lonvaud-Funel, 2001).Some LAB possesses high resistance to gastrointestinal stress and they have adhesive properties that allow them to colonize the intestinal tract (Greene et al., 1994). So the detection of BA production is dependent on increase in pH.BA production may also offer a way of obtaining energy. This function is important for microorganisms, such as lactic acid bacteria (LAB), which lacking a respiratory chain for generating high yields of ATP (Vido et al., 2004). A relation was observed between an acidic pH and increase in BA synthesis (Fernandez et al., 1997). Decarboxylase enzymes have an optimum pH of around 5.0 (Moreno-Arribas& Polo, 2008).Microorganisms can produce BAs by the activity of amino acid decarboxylases (Santhos, 1996). The groups of microorganisms including family *Enterobacteriaceae, Micrococcaceae, Pseudomonas spp., Bacillus* spp., and many LABS can synthesize amino acid by exogenous decarboxylases released (Halasz et al., 1994). The acidity within the human stomach during digestion is in the range pH1.3-3.5 which corresponds to the range of maximum concentrated activity of pepsin (Marieb et al., 2009). However, during food ingestion and depending on the food matrix, bacteria can be uncovered to a broader pH gradient. Therefore, during gastric treatment the bacteria were exposed to a decreasing range of pH from 5.0 to 1.8, which we have used for testing of probiotic and lactic acid bacteria (Fernandez et al., 2011).

Human takes only minor amounts of the polyamines spermidine, spermine and putrescine may be desirable under specific physiological conditions (Bardoa, 1993) .Low levels of biogenic amines in food are not considered a serious risk. However, if the amount consumed is high enough, or normal pathways of amine catabolism are inhibited, various physiological effects, such as hypotension or hypertension, nausea, headache, rash, dizziness, cardiac palpitation and emesis, respiratory distress, hot flushes, sweating, heart palpitation and even death may occur (Rawles et al.,1996). Biogenic amines are also considered as precursors of carcinogens, such as N-nitrosamines, and they are also an indicator of food quality (Mietz and Karmas, 1997).

Lactic acid bacteria (LAB) are widespread in nature and have been used as starter cultures in various fermented food such as fermented dairies and fermented meat products. LAB plays an important role in the fermentation process by rapid acidification of raw materials through the production of organic acids (Leroy and De Vuyst, 2004). Suitable characteristics of LAB are (a) their ability to produce antimicrobial compounds (bacteriocins, organic acids) for growth inhibition of harmful bacteria, (b) their ability to resist high concentrations of salts (in food), acids, and bile salts (in gastrointestinal tract), which is beneficial to health.

Functional food is a food, where new ingredients or more of existing ingredients have been added to a food and the new product has an additional function which relate to health-promotion or disease prevention. Potential of functional foods are to moderate disease, sponsor health and reduce health care costs. Functional foods may be whole, fortified, enriched, or improved foods.

Phenols and polyphenols and are two compounds found naturally in plants. Tea has one of the highest contents of flavonoids among common food and beverage products. Catechins in tea include EGCG (epigallocatechin-3-gallate), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), catechin, and gallocatechin (GC). Catechins contain about 25% of the dry weight of fresh tea leaf. They are present in nearly all teas made from *Camellia sinesis*, including white tea, green tea, black tea and oolong tea. Tea flavonoid consumption has been linked to lower incidences of chronic diseases such as cardiovascular disease and cancer. The catechin family of compounds are very strong therapeutic candidates for protection against the cognitive decline caused by HIV. Epicatechin, epigallocatechingallate (EGCG) and other catechin flavonoids protect against neurotoxic oxidative stress and caused by the HIV-Tat protein. To produce green tea, the young leaves are rolled and steamed to minimize oxidation. White tea is prepared from very young tea leaves or buds covered with tiny, silvery hairs, which are harvested only once a year in the early spring. White tea is steamed and they were dried immediately after picking to prevent oxidation, giving it a light, delicate taste.

Review of Literature

BAs are mainly formed in foods by microbial decarboxylation of amino acids and transamination of aldehyde and ketones (Askar et al., 1986).BAs are classified into two types according to their amine contents such as mono and poly amines (PA). The monoamines are histamine (HI), tyramine (TY) and tryptamine (TR) and polyamines are putrescine (PUT) and cadaverine (CAD). Chemical structures of some BA are given in fig1.

Every organ of the body requires PAs for its growth, renewal and metabolism as it involves in synthesis of nucleic acid and protein in each step (Shalaby, 1996). Hence, the requirement of PAs increases rapidly in growing tissues but the unnecessary intake of PA causes tumor growth. To limit the intake of PA is one of the directions in cancer therapy (Moinard et. al., 2005). The BAs which were present in foods are histamine, putrescine, cadaverine, tyramine, tryptamine, beta-phenylethylamine, spermine, and spermidine etc. Putrescine and cadaverine inhibit intestinal diamine oxidase and histamine-N-methyltransferase which metabolize histamine, resulting in an increase of histamine toxicity (Stratton et al., 1991). Furthermore, putrescine (Bills et al., 1973), cadaverine (Warthesen et al., 1975), spermidine (Bills et al., 1973; Smith, 1980), spermine and agmatine (Smith, 1980) are found to be potentially carcinogenic by converting to nitrosamine.

Table 1 shows the chemical properties of the most important BAs occurring in foods (Histamine, putrescine, cadaverine, tyramine, tryptamine, beta-phenylethylamine, spermine, and spermidine) such as molecular structure, molecular weight (http://pubchem.ncbi.nlm.nih.gov) pK value.

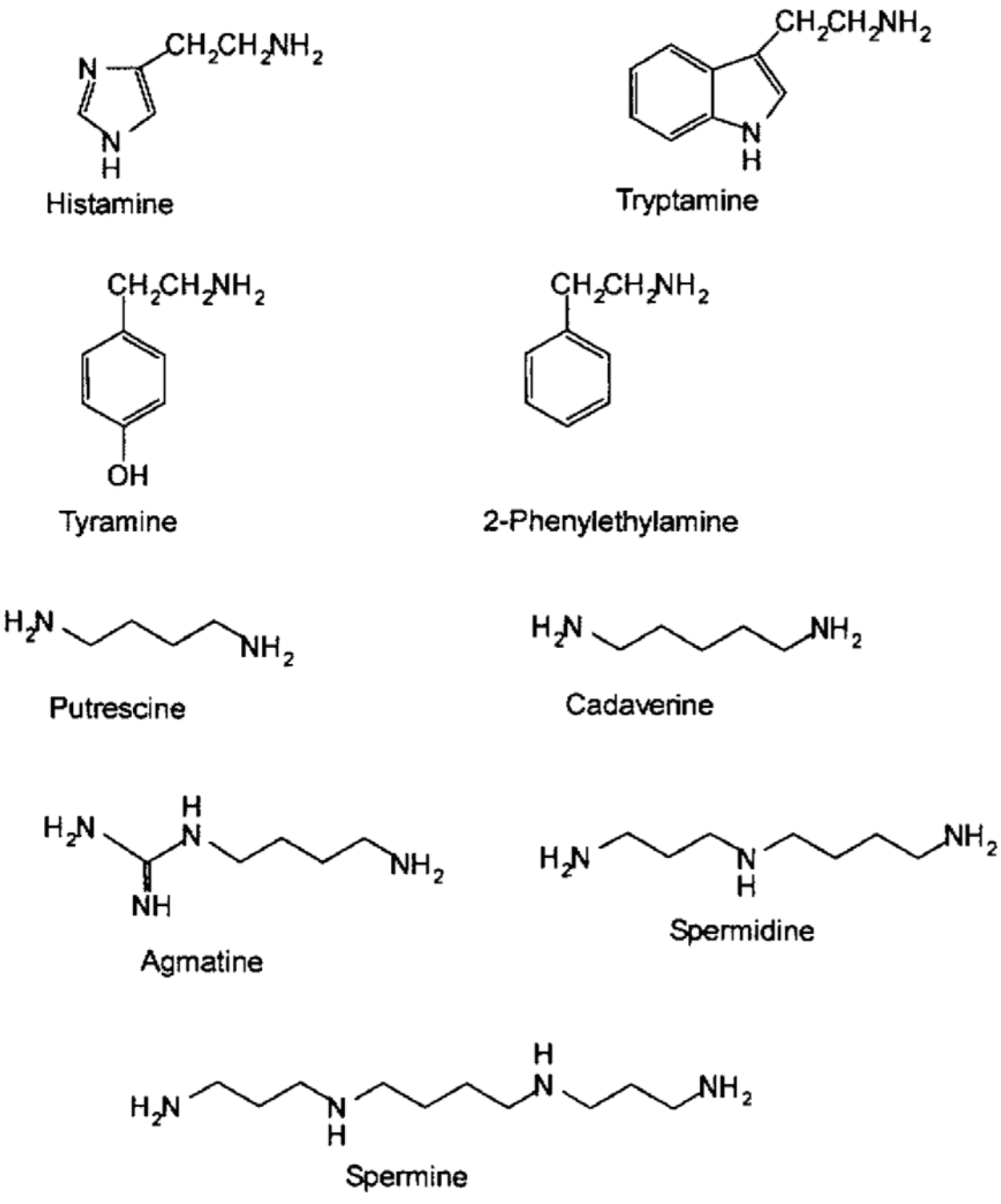


Fig. 1. Chemical structures of some biogenic amines (Adapted from Onal, 2006, Food chemistry, Science direct)

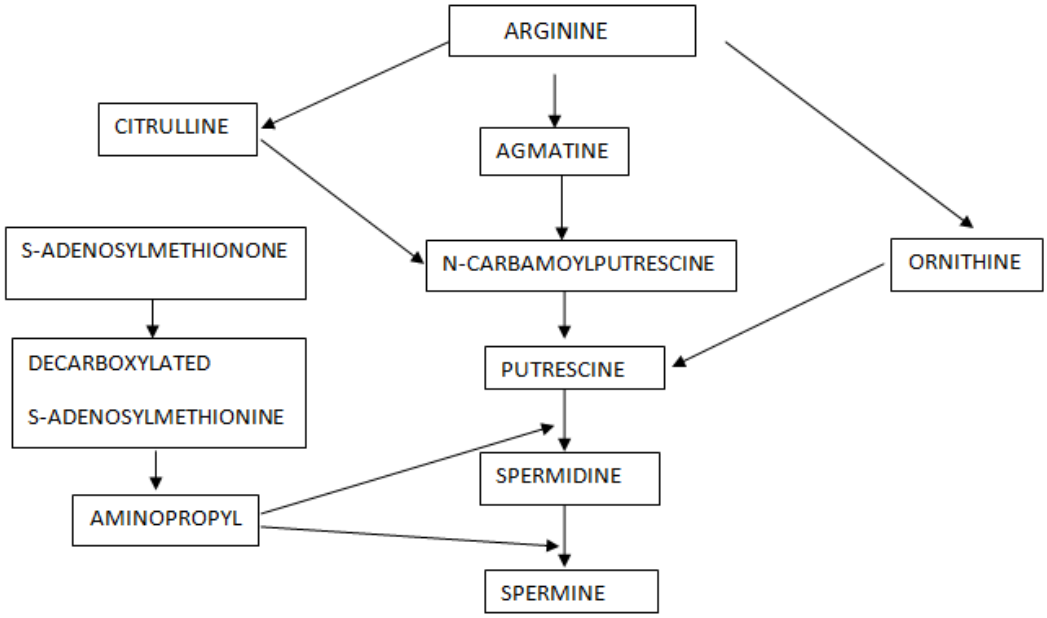


Fig. 2. Biosynthesis of polyamines (Lima &Glo´ ria, 1999)

Table: 1Chemical properties of biogenic amines a http://pubchem.ncbi.nlm.nih.gov

NAME	**ABBREVIATION**	**MOLECULAR FORMULA**	**pKb**	**MOLECULAR WEIGHT**
Tyramine	TYR	$C_8H_{11}NO$	pK = 9.6	137.2
Tyrptamine	TRP	$C_{10}H_{12}N_2$	pK = 10.2	160.2
Putrescine	PUT	$C_4H_{12}N_2$	pK_1 = 10.8, pK_2 = 9.4	88.2
Histamine	HIS	$C_5H_{10}N_3$	pK_1 = 9.8, pK_2 = 6.0	111.1
Cadaverine	CAD	$C_5H_{14}N_2$	pK_1 = 11.0, pK_2 = 9.9	202.2
Phenylethylamine	PEA	$C_8H_{11}N$	pK = 10.0	121.2
Spermine	SPM	$C_{10}H_{26}N_4$	pK_1 = 11.50, pK_2=10.95, pK_3 = 9.79, pK_4 = 8.90	202.3
Spermidine	SPD	$C_7H_{19}N_3$	pK_1 = 9.5, pK_2 = 10.8, pK_3 = 11.6	145.3
Agmatine	AGM	$C_5H_{14}N_4$	pK_1 = 12.5	130.2

The bases like histamine, serotonin, dopamine, and tyramine have important role in biological activity (Shalaby, 1996). Secondary amines like putrescine and cadaverine have an important role in food poisoning as they have the potentiality for the toxicity of histamine (Bjeldanes et. al., 1987). The quantity of BA is also considered as a marker, for level of microbiological contamination in food (Leuschner et. al., 1999). Therefore, it is necessary to monitor the biogenic amine levels in food.

Biogenic amines also formed during storage or processing of the products and are produced by thermal or bacterial enzymatic decarboxylation of free amino acids by *Bacillus*, *Clostridium*, *Hafnia*, *Klebsiella*, *Morganellamorganii*, *Proteus*, *Lactobacillus* such as *Lactobacillus buchneri* and *Lactobacillus delbrueckii* in cheese, *Enterobacteriaceae* and *Enterococcus* increasing on fish, meat and their products (Halasz et al., 1994).Synthesis of biogenic amines are shown in Fig.3.The monoamines are histamine (HI), tyramine (TY) and tryptamine (TR) which arise from histidine, tyrosine and tryptophan, respectively. Similarly, the diaminesputrescine (PUT) and cadaverine (CAD) are produced from ornithine and lysine, respectively as shown in Fig 2. Putrescine is a pioneer for the formation of polyamines, spermidine (SPD) and spermine (SPM).

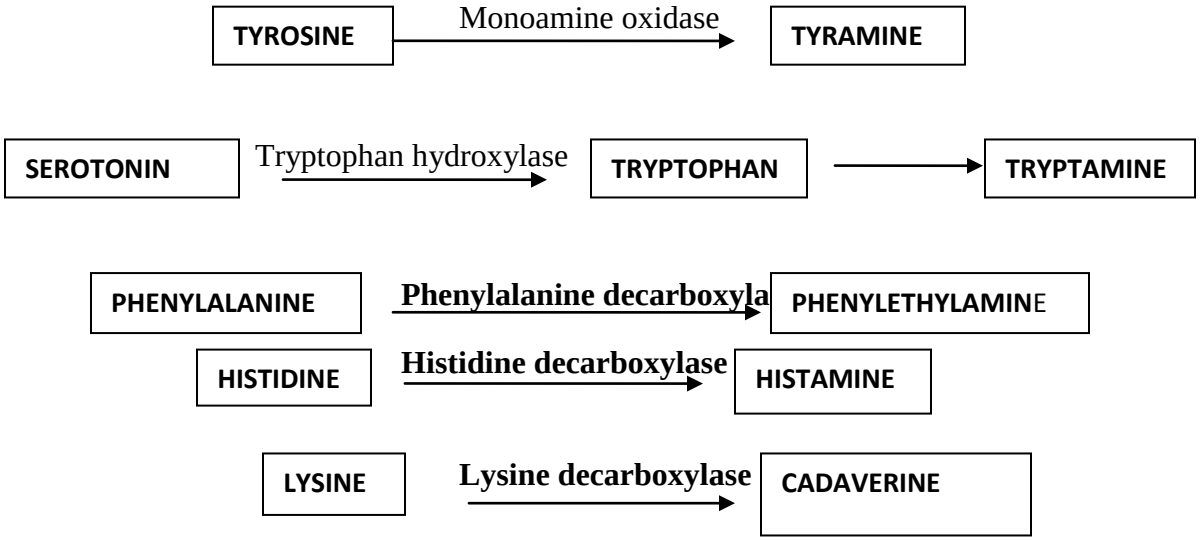


Fig. 3. Synthesis of biogenic amines (Halasz et al., 1994)

Aromatic amines like tyramine, tryptamine, and beta-phenylethylamine have a vasoconstrictor action while others (histamine and serotonin) present a vasodilator effect. But tyramine and histamine act as hormonal mediators in both humans and animals. Psychoactive aminessuch as dopamine and serotonin which act as neurotransmitters in the central nervous system (Brodo' cz, 1993). Some biogenic amines can react with nitrite and can generate carcinogenic nitrosamines (Warthese et. al., 1975). Except, their toxic effects, biogenic amines are responsible for food hygiene. High concentrations of such amines may be found in food due to use of poor quality raw materials, contamination and unsuitable conditions for food processing and storage (Brink et.al., 1990; Halasz et.al., 1994). Histamine formation is inhibited by salting with respect to concentration which is thermally stable during the cooking process. The availability of oxygen also has an important effect on biosynthesis of amines. The food rich in histamine causes allergy-like symptoms. Tyramine is identified as a major mutagen precursor.

Indigenous BA compounds are also naturally produced in different human tissues because of their biological role in processes such as synaptic transmission, blood pressure control, allergic response, and cellular growth control (Russo et al., 2010). The gastro-intestinal tract which is a function of dietary intake of food containing BA represents an exogenous source of these molecules for humans. This exogenous source of BA can incite high levels in the human organism, and in reason of their importance in physiological processes, with negative consequences to human health (Ladero et al., 2010; Russo et al., 2010). Human sensitivity fluctuates according to the correct functioning of the detoxification systems, since biogenic amines are metabolized in the human gut through the action of amine oxidases (Spano et al., 2010). This system has a protective role against the absorption of dietary BA under physiological conditions. For this reason, inan individual with a pathological deficiency of amine oxidases activity, the ingestion of food products containing excess of BA may lead to high levels of BA in the organism (Kuefner et al., 2004).

Histamine:

Askar and Treptow (1993) suggested that histamine, at a concentration of 500 mg/kg will be hazardous for human health. High levels of histamine in foods can have important vasoactive effects in humans (Lehane andOlley, 2000). Histamine-rich food also causes allergy-like symptoms (Askar andTreptow, 1993). The intestinal diamine oxidase and histamine-N-methyltransferase which metabolize histamine, resulting in an increase of histamine toxicity can be inhibited by putrescine and cadaverine (Stratton et.al., 1991).Histamine can also be detoxified by methylation (through the action of methyltransferase) or acetylating (Lehane and Olley, 2000).

Tyramine:

Consumption of high levels of tyramine has adverse effects on human health, causing migraine headaches and a sudden rise in blood pressure .Tyramine has also been identified as the major mutagen precursor (Ochai et al., 1984).But, Brink et al. (1990) reported that 100–800 mg/kg of tyramine and 30 mg/kg of N-phenylethylamine in foods are toxic, although the optimum levels for other amines are not established yet.

Other compounds:

Furthermore, putrescine (Bills et.al., 1973; Warthesen et al., 1975), cadaverine (Warthesen et al., 1975), spermidine (Bills et al., 1973; Smith, 1980), spermine and agmatine (Smith, 1980) are reported to be potential carcinogen as they get converted into nitrosamine. Mostly, biogenic amines are pharmacologically active, but oral administrations of amines do not generally provoke adverse reactions, because amine oxidases in the intestine are responsible for detoxification of these compounds (Askar&Treptow, 1986). However, food toxicity may occur (Joosten& Nun ez, 1996) due to the over saturation of amine-metabolizing activity in human body, due to an ingestion of high dose of amines and/or an impairment of metabolic activity in the presence of specific inhibitors (Taylor, 1986). In foods BAs are of great interest not only due to their toxicity, but also used as good indicators of spoilage (De Borba and Rohrer, 2007). Secondary amines, such as putrescine and cadaverine, are good indices of spoilage of marine fish (Mietz& Karmas, 1977) and they can potentiate the toxicity of histamine and react with nitrites to form nitrosamines.

The production of BAs by lactic acid bacteria is not a desirable property (Linares et al., 2011). The presence of BA in fermented food has usually been used as an indicator of starter quality. Biogenic amine production by bacteria was shown to be strain-dependent rather than being related to species specificity (Garai et al., 2007). It is important to note that it is not always possible to make such modifications to the fermenting process without causing changes in texture flavour or quality.

Functional foods

The term ‘functional foods’ consist of some bacterial strains and products of plants and animal origin containing physiologically active compounds beneficial for human health and reducing the risk of chronic disease.

According to definition, functional food is a part of diet in everyday’s food and is confirmed to offer health benefits and to reduce the risk of chronic disease beyond the widely accepted nutritional effects (Hasler, 1998). All foods are functional because all foods provide taste, aroma and nutritive value. The term functional food was first introduced in Japan in mid 1980’s. This type of food is known on Japanese market as ‘Food for specified health use’ (FOSHU). The functional food comprises of:

- Conventional foods containing naturally occurring bioactive substances (e.g., dietary fibre).
- Food enriched with bioactive substances (e.g., probiotics, antioxidants).
- Synthesized food ingredients introduced to traditional foods (e.g., prebiotics).

Among the functional food components, probiotics and prebiotics, soluble fibre, omega-3-polyunsaturated fatty acids, conjugated linoleic acid, plant antioxidants, vitamins and minerals, some proteins, peptides and amino-acids, as well as phospholipids are most important. There are numerous health effects related to functional food consumption, such as reduction of cancer risk, improvement of heart health, stimulation of immune system, decrease of menopause symptoms, improvement of gastrointestinal health, maintenance of urinary tract health, anti-inflammatory effects, reduction of blood pressure, maintenance of vision, antibacterial and anti-viral activities, reductions of osteoporosis and anti-obese effect.

Tea, in the form of green or black tea, is one of the most broadly consumed beverages in the world (Thangapazham et al., 2007). It is believed to be second only to water. Black tea is consumed in Western countries, along with some Asian countries, whereas green tea is consumed mostly in China, Japan, India, and a number of countries in north Africa and the Middle East.Green tea comes from the mature leaves of the plant and is sold as either fresh or dried unfermented leaves. The preparation of black tea requires fermentation. During this process, catechins in black tea are partly converted to theaflavins. Many health benefits have been described to consumption of this beverage, including the effects of reduction of cholesterol, defense against cardio-vascular disease and cancer (Zuo et al., 2002). All beneficial effects of tea have been attributed to the strong anti-oxidative activity of the tea phenolic compounds, known as tea catechins. Tea catechinshave strong antioxidant properties, i.e. they may protect the body from damage caused by free radical-induced oxidative stress (Manzocco et al., 1998). In addition, many reports (Chou et al., 1999) have presented data regarding the antimicrobial activity of different types of tea extracts on various pathogenic microorganisms. Therefore, the consumption of tea has been related with reduced risk of major diseases, including coronary heart disease, stroke and cancer (Benzie et al., 1999). These pharmacological have been mainly attributed to catechins (Zuo et al., 2002).Green tea is also believed to reduce arthritis and stress.In addition, it is also found to exhibit antiviral properties, anti-carcinogenic effects bone density and ultraviolet skin protection from UV rays.

In some studies using yogurt, individual LAB species, or both, promising health benefits were found for individuals with:

- Lactose Intolerance
- Constipation

- Diarrheal diseases
- Colon Cancer
- Inflammatory Bowel Disease (IBD)
- *Helicobacter pylori* infection

So tea has an antimicrobial activity against a large spectrum of pathogenic bacteria (Chou et al., 1999), and its addition to milk before fermentation would protect the final product against pathogenic or undesirable bacteria.

There are many components in foods that are supposed to have health benefits. Some examples are listed in Table 2.

Table 2: Potential Benefits of Food Components

COMPONENTS	**PRODUCT**	**POTENTIAL BENEFIT**
Lycopene	Tomato products	Reduce the risk of prostate cancer
Beta-glucan	Oats, Barley	Reduce risk of cardiovascular disease, lower LDL and total cholesterol
Long chain omega-3 fatty acids-DHA/EPA	Fish oils	Reduce risk of cardiovascular disease and improve mental functions
Catechins	Tea	Neutralize free radicals and reduce risk of cancer
Isoflavones	Soy-based products	Reduce risk of cardiovascular disease and lower LDL and total cholesterol
Flavones	Flax seed	Neutralize free-radicals and reduce risk of cancer

OBJECTIVES OF THE PROJECT

The main objectives are -

- Screening for biogenic amine producing *Lactobacillus* spp.
- Test for acid tolerance
- Development of black and green tea curd.
- pH, %of lactic acid and number of bacteria of black and green tea curd

MATERIALS AND METHODS

3.1. Isolation of bacterial culture

LAB counts in each sample were analyzed by spread plating the serially diluted homemade curd and OMFED curd samples onto MRS (de Man Rogosa Sharpe) agar. After incubation at 37°C for 48 hrs, distinct colonies were selected and isolated on MRS agar. The name of the strains was OLAB-W, OLAB-S, OLAB-F, HLAB-W and HLAB-C.

3.2. Activation of microbial culture

In order to promote the enzyme induction before the actual screening test, *Lactobacillus* strains were sub-cultured 5 to 10 times in MRS broth.

3.3. Morphological identification by Simple Staining method

For simple staining method, methylene blue dye was used for 60 seconds. It was observed under microscope using oil immersion objective.

3.4. Screening method

The composition (%) of decarboxylase media is described below

Components	Concentrations(g/litre) media
Tryptone	0.5
Yeast extract	0.5
Meat extract	0.5
Sodium chloride	0.25
Glucose	0.05
Tween 80	0.1
Magnesium sulphate ($MgSO_4$)	0.02
Manganese sulphate ($MnSO_4$)	0.005
Ferrous sulphate ($FeSO_4$)	0.004
Ammonium citrate	0.2
Thiamine	0.001
Potassium phosphate(K_2PO_4)	0.2
Calcium carbonate ($CaCO_3$)	0.01
Pryridoxal-5-phosphate	0.005
Amino acid	1.0
Bromocresol purple	0.006
Agar	2.0
PH	5.3

Glucose concentration was reduced to 0.05% in order to avoid excessive acid production that might counteract the pH increase by the BA formation. As a result, salt concentration was slightly lower. Metal sulphates, such as Mg (0.02%), Mn (0.005%), and Fe (0.004%), Tween 80 (0.1%), and ammonium citrate were included to enhance the growth of LAB. Since some LAB strains (especially hetero-fermentative LAB) require thiamine to grow, 0.001% of this vitamin was added.0.2% of *di*-potassium phosphate and 0.01% of calcium carbonate was taken In order to improve the buffer effect and to neutralize the acid produced in comparison with *di*-potassium phosphate. Also, pyridoxal-5- phosphate was included to the decarboxylase medium (at 0.005%) since its presence as a cofactor for the decarboxylation reaction has a strong enhancing effect on the amino acid decarboxylase activity. The concentration of each amino acid was 1%. Bromocresol purple was used as pH indicator at 0.006%. The pH was adjusted to 5.3 and the medium was autoclaved for 10 min at 121°C to avoid excessive hydrolysis of agar at the low pH. Wells of diameter of 0.5 cm were made in all the decarboxylase plates and all the above mentioned microbial cultures were inoculated into the wells.

3.5. Confirmation of biogenic amine formation

Confirmation of the amine-forming capacity of each microbial culture was performed through a qualitative chemical analysis of the BA (tyramine, histamine, putrescine and cadaverine) potentially formed in the fermenting broth. The strains, previously cultured in the MRS or Nutrient Standard broth (with the precursor amino acids and pyridoxal-5-phosphate added), were inoculated at 0.1% into a decarboxylase broth, formulated as the screening medium but without agar and containing 0.5% of tyrosine, 0.25% of histidine, ornithine and lysine. Then they were incubated at 37°C for 24 hrs under aerobic condition. Microbial cultures which were not producing biogenic amines were selected for further studies.

3.6. Acid tolerance test

Active cultures were centrifuged at 5000rpm for 10 minutes at 37°C.Pellets were washed in PBS (phosphate saline buffer) at pH 7.4. Pellets were resuspended in PBS having pH 7.4, 5.5, 4, 3 and2.Then cultures were spread over the MRS agar plates. The plates were then incubated at 37°C. From these plates survival of bacteria was checked and the tolerant types were identified.

3.7. Preparation of tea curd

2% of green tea and black tea was added in milk and boiled. Then they were kept for 5 minutes for extraction with shaking and filtered and cooled. The negative bioactive amine strains were inoculated in prepared green tea and black tea bottles. Then the bottles were kept at temperature 45°C for 12 hours.

3.8. pH of the curd

The pH of the green and black tea curds were determined by using pH meter.

3.9. Total bacteria in green and black tea

The green and black tea curds were serially diluted and spread over MRS agar plates. Then these plates were incubated for 24 hours at 37°C.

Number of bacteria in 1 ml of black/green tea curd = Total colonies× Dilution factor

4.0. Total acidity measurement of curd

10ml of distilled water was added with 10ml of green/black tea curd and boiled to drive out CO_2.Then they were cooled and 5 drops of 0.1% phenolphthalein were added to them. Then they were titrated with 0.1N NaOH until they became pink in colour.

% of lactic acid =ml of alkali× normality of NaOH×9/weight of sample (g)

RESULTS AND DISCUSSION

1. Isolation of Probiotic microbes from fermented foods

Isolation was done from Home made and Omfed curd (Omfed Dairy Industry, Rourkela, Odisha)

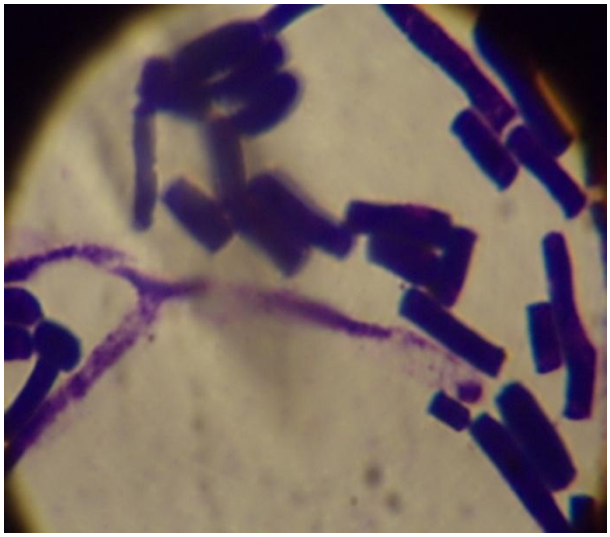


Fig-4 OLAB-W

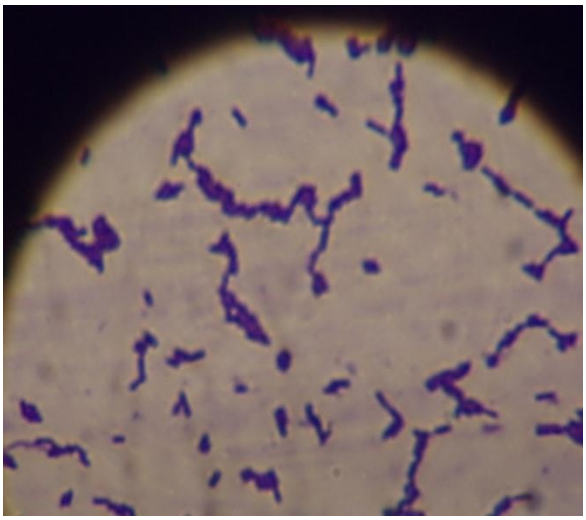


Fig- 5 OLAB-F

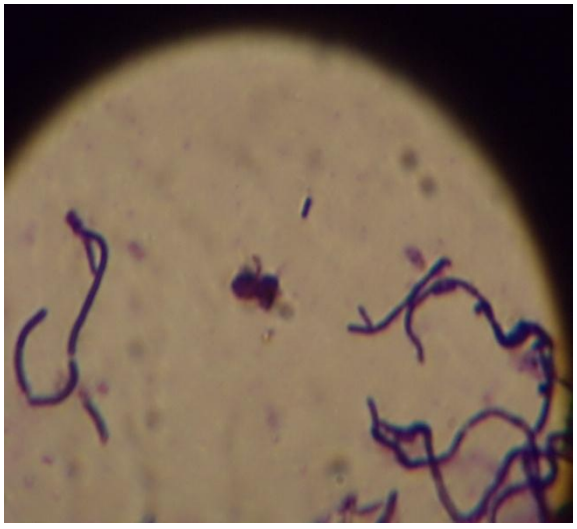


Fig-6 OLAB-S

OLAB-W, OLAB-F, OLAB-S bacterial strains were isolated from OMFED curd(Omfed Dairy Industry, Rourkela, Odisha) as depicted in Fig- 4, Fig- 5 and Fig- 6.

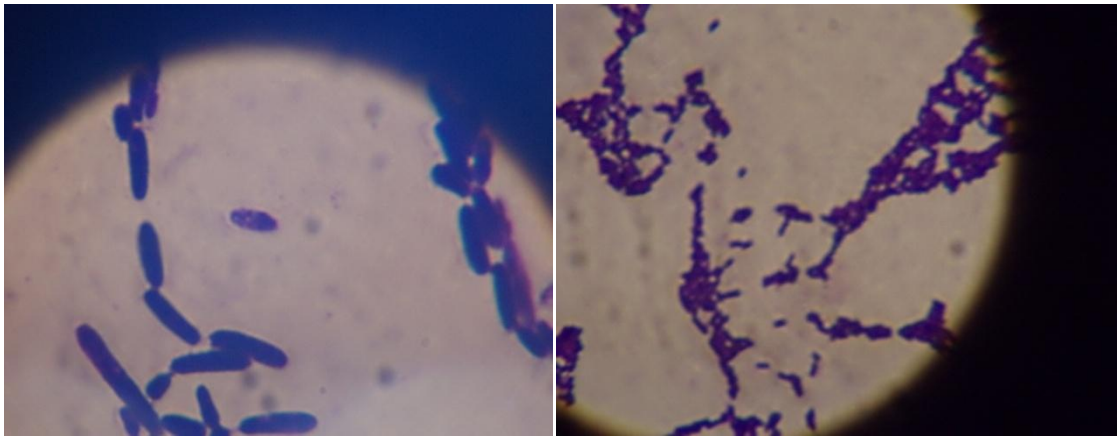


Fig-7 HLAB-C Fig- 8 HLAB-W

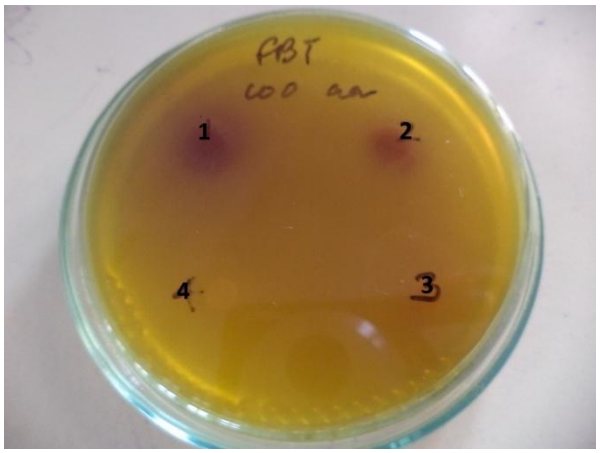
From Fig-7 and Fig-8, HLAB-C and HLAB-W bacterial strains were isolated from home made curd.

2. Morphological characterization by simple staining

STRAINS	MORPHOLOGY	BACTERIA/YEAST
OLAB-W	**Long rod**	**Bacteria**
OLAB-C	**Oval shape**	**Yeast**
OLAB-F	**Long rod**	**Bacteria**
OLAB-S	**Long rod**	**Bacteria**
HLAB-W	**Long rod**	**Bacteria**
HLAB-C	**Long rod**	**Bacteria**
HN-W	**Oval shape**	**Yeast**
HN-C	**Oval shape**	**Yeast**

From this table OLAB-W,OLAB-C,OLAB-F,OLAB-S,HLAB-W,HLAB-C,HN-W and HN-C culture strains were taken and numbered as 1,2,3,4,5,6,7and 8. From these strains OLAB-C, HN-W, HN-C were excluded as they were not bacterial strains.

3. Screening of decarboxylase activity in control



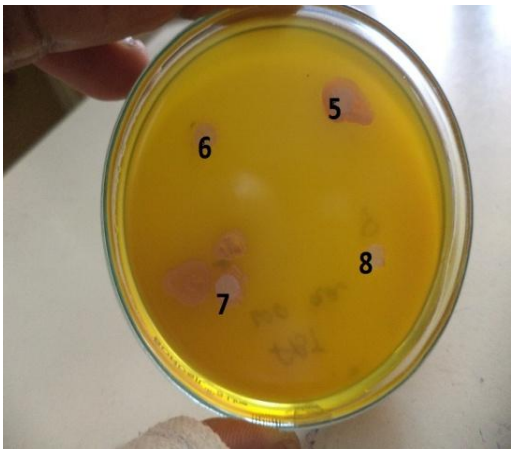


Fig-9 **Fig-10**

OLAB-W, OLAB-C, HLAB-W and HN-W showed purple colour zones as they were biogenic amine positives as depicted in Fig-9 and Fig-10. But OLAB-C and HN-W were yeasts which were not considered here. So OLAB-W and HLAB-W *Lactobacillus* strains were biogenic amine positives. These were control plates lacking the amino-acids. But it gave purple colour because of amino acid present in tryptone, yeast extract and beef extract. It was used to compare the purple colour with other plates with different amino-acids.

3.1. Decarboxylase media with tyrosine

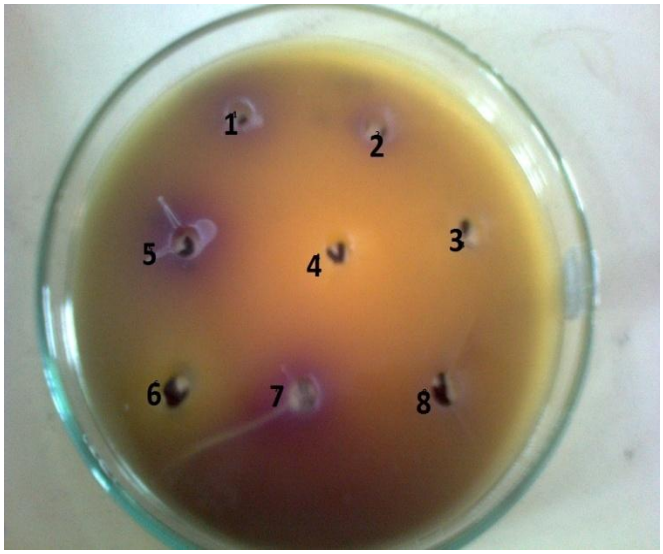


Fig-11

The strains designated number1, 2, 5, 7 number strains gave purple colour zones. So these were biogenic amine positives. Although 2 and 5 were yeasts, they also showed positive results. OLAB-W and HLAB-W *Lactobacillus* showed positive results. They produce biogenic amine thyramine.

3.2. Decarboxylase media with Ornithine

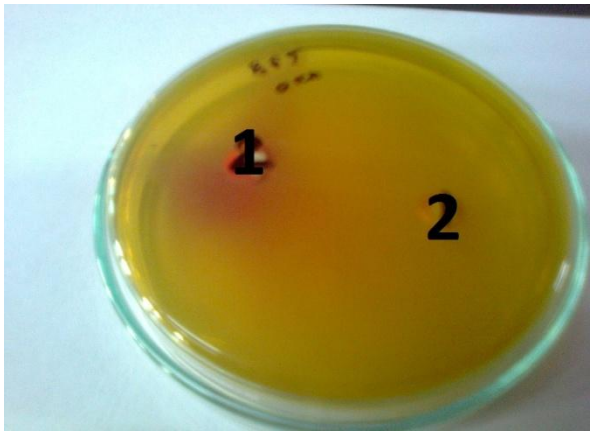


Fig-12

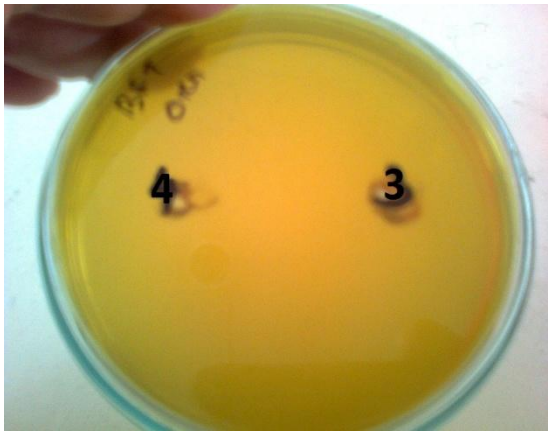


Fig-13

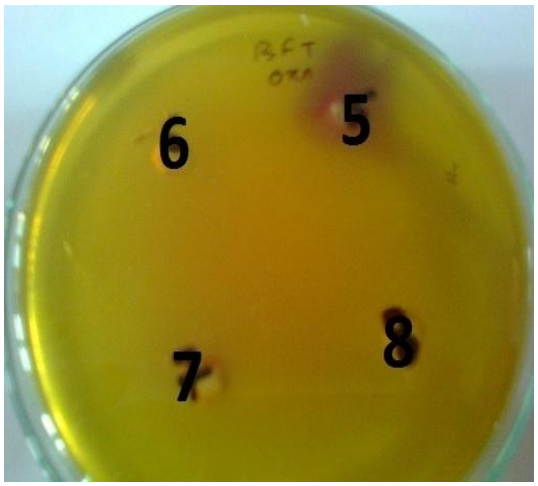


Fig-14

Positive reactions on decarboxylase plates, were recorded when a purple colour zone is seen. Here OLAB-W and HLAB-W strains were biogenic amine positives as depicted in Fig-14**.** They produce biogenic amine putrescine.

3.3. Decarboxylase media with lysine

Fig-15

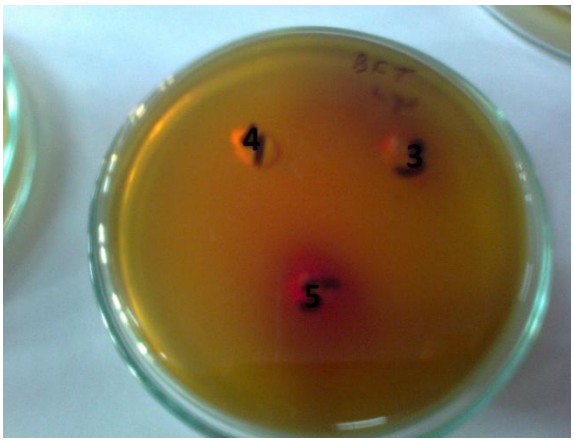


Fig-16

Fig-17

OLAB-W, OLAB-F and HLAB-W strain numbers show purple coloured zone on decarboxylase agar plateas depicted in Fig-15,Fig-16 and Fig-17. So these stains were biogenic amine positives**.** They produce the biogenic aminecadaverine.

3.4. Decarboxylase media with histidine

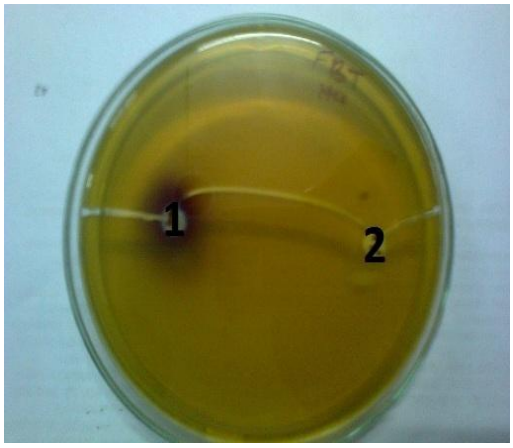


Fig-18

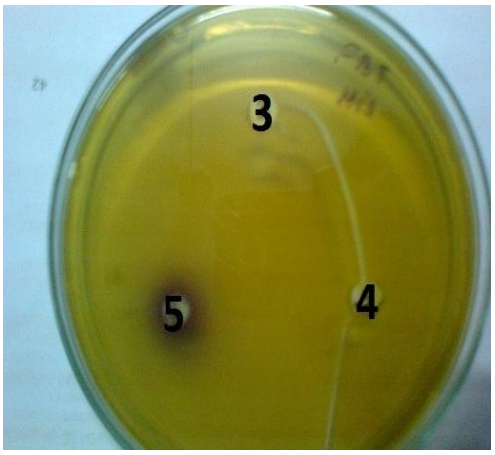


Fig-19

Yeast strains were not included here. Strains number OLAB-W and HLAB-W showed purple colour zones as shown in Fig-18 and Fig-19. So these were biogenic amine positives and produce biogenic amine histamine.

Fig-20 Confirmation of biogenic amine formation in controls

It was concluded that, in d**ec**arboxylase control broth lacking amino acid, all the stains showed negative results for biogenic amine production as depicted in Fig-20.

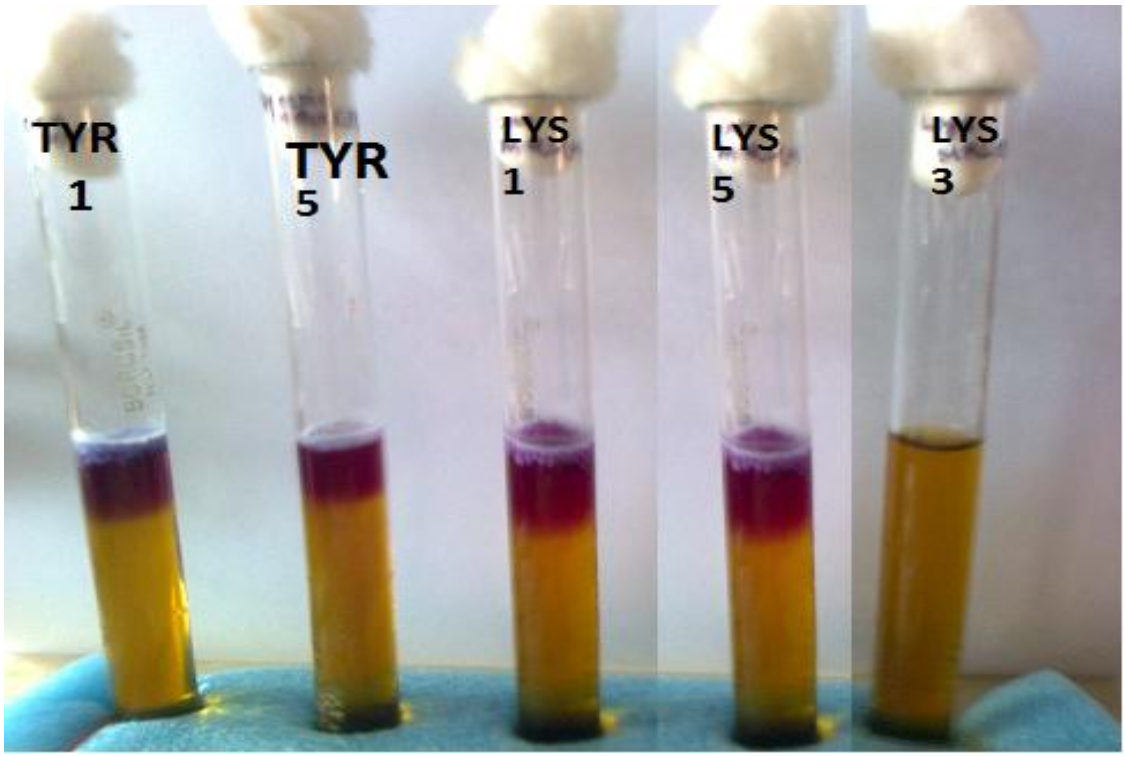


Fig-21Confirmation of biogenic amine formation in tyrosine and lysine

In decarboxylase broth supplemented with tyrosine, strain number OLAB-W and HLAB-W show purple colour. So these were biogenic amine positives. In decarboxylase broth formulated with lysine, strain numbers OLAB-W and HLAB-W gave purple colour, but OLAB-F strain did not show purple colour in decarboxylase lysine broth. So in decarboxylase agar plate it showed false biogenic amine positives as shown in Fig-21.

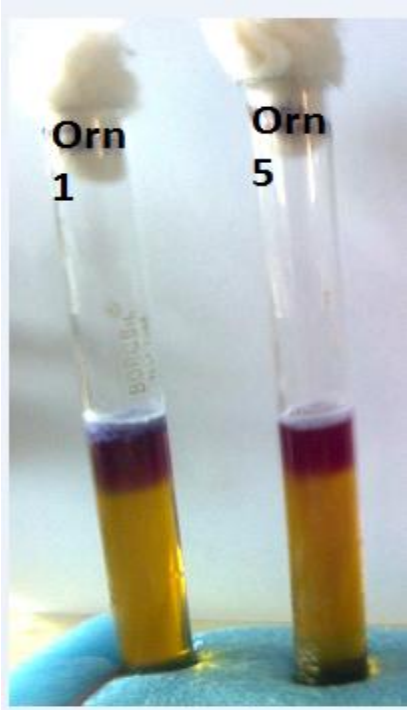


Fig-22**Confirmation of biogenic amine in Ornithine**

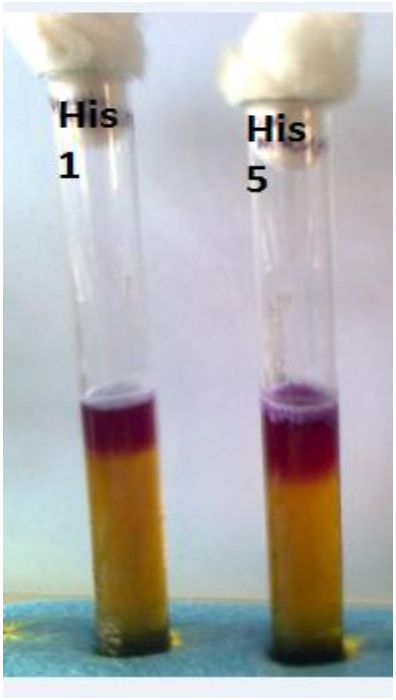


Fig-23 **Confirmation of biogenic amine in Histidine**

In decarboxylase ornithine broth, strains OLAB-W and HLAB-W gave purple colour. So these were biogenic positives as shown in Fig-22.

In decarboxylase histidine broth, strains OLAB-W and HLAB-W gave purple colour. So these were biogenic amine positives as shown in Fig-23.

Table-3 Biogenic amine positive and negative LAB results

STRAINS	TYROSINE (tyramine)	LYSINE (cadaverine)	ORNITHINE (putrescine)	HISTIDINE (histamine)
OLAB-w	positive	positive	positive	positive
OLAB-S	Negative	Negative	Negative	Negative
OLAB-F	Negative	Negative	Negative	Negative
HLAB-w	positive	positive	positive	positive
HLAB-C	Negative	Negative	Negative	Negative

4. Acid tolerance test

Table -4

Strains	pH 7.4	pH 5.5	pH 4	pH 3	pH 2
OLAB-F	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**
OLAB-S	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**
HLAB-C	GROWTH	GROWTH	GROWTH	GROWTH	**NO GROWTH**

From table-4 it can be concluded that, OLAB-F, OLAB-S, HLAB-C are biogenic amine negatives and tolerate up to pH 3.0.But in pH 2 none of the strains grew. They are used for the preparation of tea curd.

5. Preparation of tea curd

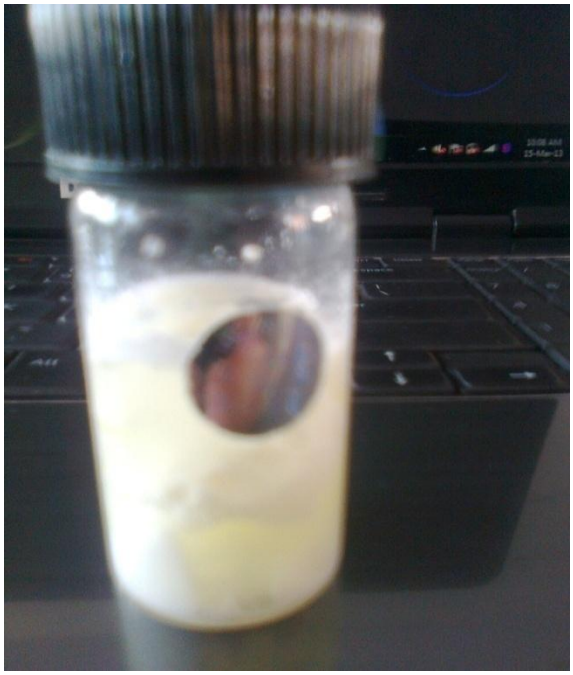


Fig-24: Green tea curd

Fig-25: Black tea curd

Remaining biogenic negative amines were inoculated in black and green tea and the temperature was maintained at 45°C. After 12 hours, black and green tea curds were prepared which were shown in Fig-24 and Fig-25.

6. pH, %of lactic acid and total bacterial colony (CFU)of the black and green tea curd

Table -5

Tea curd	**pH**	**% of lactic acid**	**Total bacterial number per ml**
Black tea curd	3.62	1.35	1.7×10^{12}
Green tea curd	3.56	1.44	1×10^{12}

CONCLUSION

Screening of non-biogenic amine producing LAB from OMFED and homemade curd was done. The bacteria having decarboxylase activity gave purple zone due to production of basic amines. Out of five strains only two were found to produce biogenic amines while the other three gave negative result. Those which did not produce biogenic amines were then utilized to prepare green and black tea curds. These curds contain health enhancing compounds such as catechins and flavonoids from tea and also the probiotic LAB strains helpful in many ways. All the three negative strains showed acid tolerance up to pH 3 while they could not survive in pH lower than that. Hence they can be successfully used in curd production as probiotic bacteria since they can tolerate the acidic pH of the upper part of gastrointestinal tract. Tea catechins have strong antioxidant properties, i.e. they may protect the body from damage caused by free radical-induced oxidative stress. And tea flavonoid consumption has been linked to lower incidences of chronic diseases such as cardiovascular disease and cancer.

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