A

DISSERTATION ON

Characterization of tannase production by *Lactococcus lactis* subp *lactis* and its potential in enhancing nutritional value of a composite sourdough

In the partial fulfillment of the requirement of the degree of

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IN

BIOTECHNOLOGY

By

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CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled **"Characterization of tannase production by *Lactococcus lactis* subsp *lactic* and its potential in enhancing nutritional value of a composite sourdough"** in the partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out during the period of one year from July 2013 to June 2014, under the guidance of Dr. Abhijit Ganguli, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization of tannase production by *Lactococcus lactis* subsp *lactic* and its potential in enhancing nutritional value of a composite sourdough"** submitted by Varsha in partial fulfillment of the requirement for the award of Degree of Master of Technology in Biotechnolgy to Thapar University, Patiala, is a record of student's own work carried out by her. 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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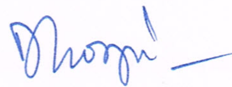

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LIST of SYMBOLS

Kg	Kilogram
mM	Milli Molar
mg	Milli Gram
ɣ	Gamma
w/v	Weight/Volume

ABSTRACT

In foods, tannins are considered nutritionally undesirable. Composite sourdough batter prepared using *vigna mungo* flour and wheat flour (Bhanwar *et al.,* 2013) makes it an attractive source for protein fortification and nutritional enrichment, however presence of antinutrients like tannins, phytic acid, saponin etc. hinder their utilization in human body. In the present study we report the tannin degradation ability of probiotic strain *Lactococcus lactis* subsp *lactis*. This strain was able to tolerate tannin concentration of 0.25 mM, tannin degradation occurred primarily by enzyme tannase and significant ($p<0.05$) degradation (44.2%) was observed at tannin concentration of 0.08mM, pH-6.8, temperature 37˚C and without agitation after 6 hours of fermentation in MRS media. Tannase production was inducible, was found to be thermostable and detected both intracellularly and extracellularly. Intracellular tannase showed higher activity in comparison to extracellular. As microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community. Therefore, we checked for biofilm development and results showed that there is more biofilm formation in presence tannin and biofilm formation was not affected due to the presence of tannin. In order to access the applicability of *L. lactis*, an experimental composite sourdough containing *Vigna mungo* and wheat flour was prepared. Fermentation was initiated by inoculating overnight culture of *L. lactis* followed by incubation at 37˚C for 4 hours. Analysis of sourdough extracts indicated a complete reduction of tannin (99.98%) the principal anti-nutrients in *Vigna mungo*. The result of this study suggests a potential applicability of *L. lactis* subsp *lactis* in degrading antinutrients, thus enhancing the nutritional value of traditional Indian composite sourdough.

Keywords: Antinutrients, Sourdough, *Vigna mungo*, Biofilm, Tannin

CHAPTER 1

INTRODUCTION

1. Introduction

Sourdough is a leaven consisting of dough in which fermentation is active. Composite sourdough prepared using *Vigna mungo* and wheat flour can serve as a good source for proteins, carbohydrate, fibre etc. Sourdough is an important modern fermentation method of cereal flours and water. Slack flour dough is inoculated with microbial starter, “mother culture”, which is constantly renewed in a cyclical way, using specified recopies and ripening conditions. Sourdough is dough which has microorganisms (for example *lactobacillus* or *yeast*) from sourdough or sourdough starters, which are active or can be reactivated. With the addition of grain products and water, they are capable of continuous acid generation (Katina *et al.,* 2005). The key groups of fermenting organisms, in addition to the yeasts, are strains of lactic acid bacteria (LAB)

Legumes like *Vigna mungo* are an important source of protein in the diets of the majority of the population in India and several other developing countries of the world. The presence of antinutrients like tannins, saponins, phytic acid and other antinutritional substances hinder their utilization in the human body. Microbial degradation of these antinutrients can present a solution for nutritional sustainability and utilization of nutrients (Rehman and Shah, 2001).

1.1 Types of sourdough

1) Type 0

When water and flour are mixed and left alone for sometime at an appropriate temperature, the mixture will get sour and start to bubble. Using this as dough to make bread is probably the most traditional and oldest way to make fermented bread. The organisms found here differ from other Sourdough Types in such that the lactobacillus bacteria are mostly homofermentative.

2) Type I

Sourdoughs grown at ambient temperatures (20 - 30 ˚C) and continous propagation (i. e.) a small amount of dough is taken from the current batch and used for the next batch. The microorganisms found in this environment are heterofermentative, mainly *lactobacillus sanfranciscensis* and dominating yeast like *Candida milleri*.

3) Type II

Sourdoughs grown with continuous propagation in an industrialized environment at higher temperature, higher hydrations and longer fermentation times (5 days). The organisms established in this environment differ from Type I and are adapted to the paramenters, like *lactobacillus pontis*, *lactibacillus panis*.

4) Type III

Sourdoughs initiated from artificially composed dried sourdoughs selected for their tolerance to drying.

1.2 Antinutrients

Substances which either by themselves or through their metabolic products, interfere with food utilization and affect the health and production of animals. Antinutrients exist in a wide range of plant foods, but they are found in particularly significant amounts in cereals and legumes, such as wheat and beans. Among the dozens of different antinutrients are enzyme inhibitors, flatus factors, saponins, and phytates. "The negative effect of antinutrients is mainly due to their effect on the absorption and utilization of nutrients,"

Phenolic compounds such as tannins are found in reasonably large quantities and in a variety of chemical forms in plant foods and serve as secondary metabolites that protect plant tissues against injuries and insect and animal attack. Phenolic compounds found in foods generally contribute to their astringency and may also reduce the availability of certain minerals such as zinc. During thermal processing, phenolic compounds may undergo oxidation and oxidized phenolics so formed, such as quinones, may combine with amino acids, thus making them nutritionally unavailable.

1.2.1 Tannin

Tannins are defined as naturally occurring water soluble polyphenols of varying molecular weight, which differ from most other natural phenolic compounds in their ability to precipitate proteins from solutions (Spencer *et al.,* 1988). Based on their structures and properties, they are distributed into two major groups – hydrolysable and condensed tannins. Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose, and are readily hydrolysed by acids or enzymes into monomeric products. On the basis of their structural characteristics it is therefore possible to divide the tannins into four major groups: Gallotannins, ellagitannins, complex tannins, and condensed tannins.

(1) **Gallotannins** are all those tannins in which galloyl units or their *meta*-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units.
(2) **Ellagitannins** are those tannins in which at least two galloyl units are C–C coupled to each other, and do not contain a glycosidically linked catechin unit.
(3) **Complex tannins** are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit.
(4) **Condensed tannins** are all oligomeric and polymeric proanthocyanidins formed by linkage of *C*-4 of one catechin with *C*-8 or *C*-6 of the next monomeric catechin.

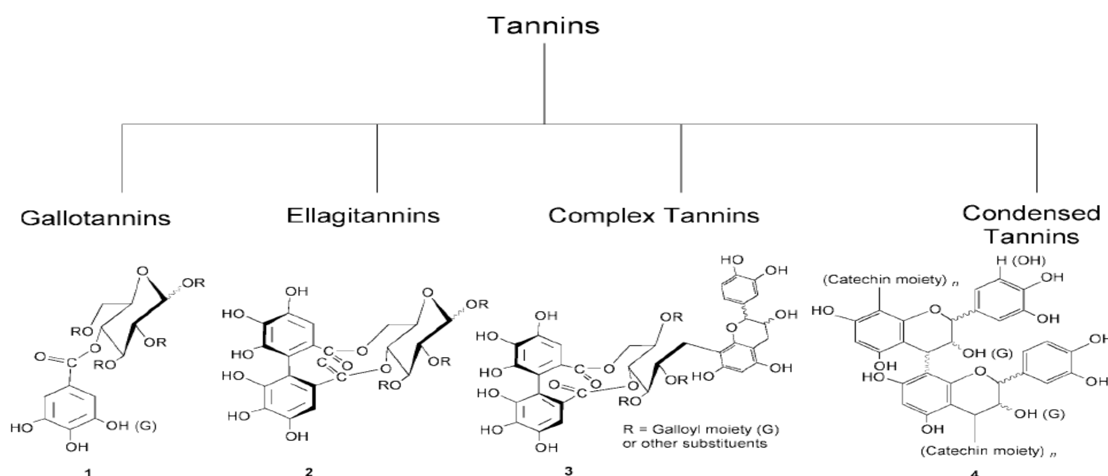

Fig. 1.1 Types of tannin on the basis of structural characteristics

1.3 *Lactococcus lactis* and its role in sourdough

Lactococcus lactis is a microbe classified informally as a Lactic Acid Bacterium because it ferments milk sugar (lactose) to lactic acid. Lactococci are typically spherical or ovoid cells, about 1.2µm by 1.5µm, occurring in pairs and short chains. They are Gram-positive, non motile, and do not form spores. Lactococci are found associated with plant material, mainly grasses, from which they are easily inoculated into milk. Hence, they are found normally in milk and may be a natural cause of souring. *Lactococcus lactis* has two subspecies, *lactis* and *cremoris*, both of which are essential in manufacture of many varieties of cheese and other fermented milk products. The metabolic pathway of *L. lactis* can function through aerobic and anaerobic reactions. The main metabolism of *L. lactis* is through the anaerobic pathway, fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production. The use of *L. lactis* in elevating the amount of GABA in composite sourdough prepared using wheat and *Vigna mungo* flour was reported by (Bhanwar *et al.,* 2013).

1.4 *Vigna mungo* and composite sourdough

Pulses are valued for their protein content as well as their low glycemic index and are commonly included in diets in the Indian subcontinent (Khandelwal *et al.,* 2010). Pulses, when blended with cereal proteins and other foods rich in sulphur containing amino acids and tryptophan, provide a well balanced essential amino acid profile and may offer a promising alternative source for nutritional and functional proteins (Boye *et al.,* 2010). Black gram (*Vigna mungo*) is an important pulse crop occupying unique position in Indian agriculture.

The seeds of black gram contain a moderately high amount of calories (calorific value of 350 cal/100 g), carbohydrates (56.6%), proteins (26.2%) and fat (1.2%t). In addition to being an important source of proteins and calories, black gram is rich in minerals — calcium (185 mg/100 g), iron (8.7mg/100 g) and phosphorus (345 mg/100 g), and vitamins — vitamin B1 (0.42mg/100 g), vitamin B2 (0.37mg/100 g) and niacin (2mg/100 g) (Panhwar, 2005).

However, apart from these health promoting components, black gram also contains a number of bioactive food components (BAFCs) which elicit physiological effects beyond those associated with essential human nutrition (BAFCs, 2008). Many of the bioactive substances have

been classified as 'Antinutritional Factors' (ANFs). Pulses contain several antinutritional factors such as flatulence causing raffinose family oligosaccharides (RFOs), protease inhibitors, saponins, tannins, lectins, phytic acid and lathyrogen which reduce nutrient utilization and intake of food (Jain *et al.,* 2009).

Phenols bind to positively charged proteins, amino acids and/or multivalent cations or minerals such as iron, zinc and calcium in foods and decrease their digestibility (Gilani *et al.,* 2005). Tannins (polyphenols) interact with both enzyme and non-enzyme proteins to form tannin–protein complexes resulting in inactivation of digestive enzymes reducing protein digestibility (Khandelwal *et al.,* 2010). In earlier studies, a combination of wheat + *vigna mungo* sourdough was reported (Bhanwar *et al.,* 2012).

However one cannot be overly optimistic about the result of the composition due to the presence of significant amount of antinutritional components in the same and little of any is known on the role of starters in degrading antinutrients in sourdough. Therefore one of the prime objectives of the experiment is to trim down the level of presence of these antinutritional components which is degrading the quality of the composition. Apart from this, these list of objectives, were framed to overall improve the quality of the content of the composition.

Objectives of the present study

i) Characterization of antinutrients degrading LAB starter culture(s).
ii) Optimization of inoculum size, culture conditions using functional starter cultures (LAB species) for degradation of target antinutrient i.e. tannin in composite sourdough batter prepared using wheat + *Vigna mungo flour.*

iii) Survival studies of LAB in the formulated food matrix.

iv) Application of functional starters in composite sourdough batter for maximal reduction of tannin and optimal fermentation of composite dough batter.

CHAPTER 2
LITERATURE REVIEW

2.1 Sourdough fermentation and nutritional quality

Sourdough fermentation can influence the nutritional quality by decreasing or increasing levels of compounds and enhancing or retarding the bioavailability of nutrients.

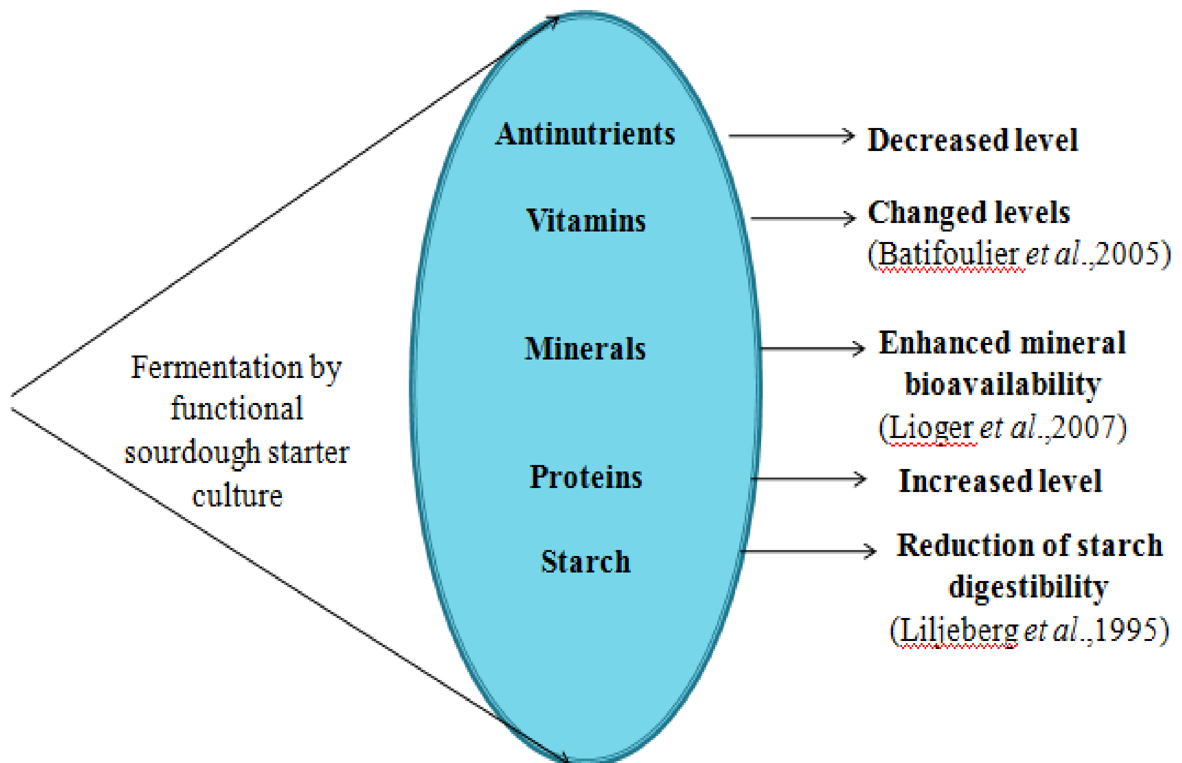

Fig.2.1.Potential mechanisms by which sourdough fermentation may influence the nutritional quality

It has been suggested that a moderate decrease of pH to 5.5 during sourdough fermentation is sufficient to reduce phytate content of whole-wheat flour by about 70% by the endogenous phytase present in the flour (Leenhardt *et al*., 2005).The fermentation step can affect the overall retention of vitamins in the baking process. A short baking process was shown to decrease the

vitamin B1 in whole-wheat baking, but a prolonged yeast or sourdough fermentation maintained it (Batifoulier *et al.,* 2005).

Mineral bioavailability - Wholemeal foods provide a good source of minerals in the diet, including calcium, potassium, magnesium, iron , zinc and phosphorous. The bioavailability of minerals may, however, be limited due to the presence of tannins and phytate, myo-inositol hexaphosphate. Contents of 3–22 mg/g phytic acid have been reported in grains (Garcia *et al.,* 1999).

Levels and stability of vitamins and bioactive compounds - Cereal foods have for long been known to be an important source of vitamins, such as thiamine, vitamin E and folates. The fermentation step can thus affect the overall retention of vitamins in the baking process. A short baking process was shown to decrease also the content of vitamin B1 in whole-wheat baking, but a prolonged yeast or sourdough fermentation maintained it. Whole-wheat breadmaking with yeast (from kneading to final bread), with long fermentations, resulted in a 30% enrichment in riboflavin. The use of mixed fermentation conditions (yeast plus sourdough) did not have a synergistic effect on B vitamin levels (Batifoulier *et al.,* 2005)

Influence of sourdough on starch digestibility – Dietary carbohydrates represents a major source of plasma glucose. An increase in the amount of rapidly digestible carbohydrate in the diet increases blood glucose levels. Consequently, many common starchy foods like bakery goods, breakfast cereals, potato products and snacks produce high glycemic responses. The fermentation of wheat and rye flour matrix with lactic acid bacteria (sourdough process) has been shown to lower GI of wholemeal barley bread (Liljeberg *et al.,* 1995) and wheat bread (De Angelis *et al.,* 2006; Maioli *et al.,* 2008), and insulin index (II) of rye breads with varying fibre content (Juntunen *et al.*, 2003). Several mechanisms have been proposed for the ability of sourdough processing to reduce starch digestibility. The effect is assumed to be mainly due to formation of organic acids, especially lactic acid, during fermentation. The physiological mechanisms for the acute effects of acids appear to vary; whereas lactic acid lowers the rate of starch digestion in bread (Liljeberg et al., 1995), acetic and propionic acids appear instead to prolong the gastric emptying rate (Liljeberg and Bjorck, 1998).

Potential of sourdough in fibre enriched products

Sourdough provides aromatic and pleasing flavour, and improves overall quality and shelf life of whole grain breads. Another example of the potential of sourdough is the ability to modify the bran fraction of the grain (rich in fibre) in such a way that larger amounts of bran can be utilised in breads.The nutritional importance of DF has been demonstrated in many studies. A typical Western diet contains less than 20 g/day, whereas the recommended daily intake is 25–30 g (Cummings & Frolich, 1993). Thus, at the moment most people eat too little fibre and these low levels of DF in Western diet contribute to a long list of diseases, ranging in severity from dental caries through constipation to obesity, colorectal cancer, coronary heart disease and type 2 diabetes.

According to Seibel (1983) addition of fibre causes the following technological changes:

(i) Increases dough yield

(ii) Results in a moister and shorter dough

(iii) Decreases fermentation tolerance (that is dough is able to keep the optimum volume a shorter time during proofing)

(iv) Decreases bread volume

(v) Creates a crumb which is tense and non-elastic

(vi) Creates flavour changes depending on type of fibre and bread type.

2.2 Biochemistry and physiology of sourdough Lactic acid bacteria

Sourdoughs are very complex biological ecosystems since the microbial composition and the interactive effects among bread making processes and ingredients (Gobbetti, 1998). As a general rule, LAB are the dominant organisms in sourdoughs and in many cases they co-exist with yeasts which are also present in elevated numbers (Vogel et al., 1999). Bacteria use carbon sources in a strictly controlled hierarchical manner for which they have developed global control mechanisms (Titgemeyer & Hillen, 2002).

Overall, sourdough fermentation with LAB resulted in an increase of amino acid concentrations during fermentation, whereas dough fermentation with yeasts alone reduced the concentration of free amino acids. This enhanced proteolysis is attributed either to the proteolytic

activity of sourdough LAB and/or to an activation of proteolysis by cereal enzymes under the acidic conditions of sourdough fermentation (Gobbetti, 1998; Gobbetti *et al.,* 1994).

Anti bacterial activity - Bread and other leavened baked products can become contaminated with spoilage bacteria or moulds. LAB has been shown to possess both anti-bacterial and anti-fungal properties and sourdough addition is an effective procedure to preserve bread from spoilage since it complies with the consumer request for additive-free products (Messens & De Vuyst, 2002). Besides various compounds (e g. organic acids, hydrogen peroxide, diacetyl), sourdoughLAB can inhibit the growth of other, usually related microorganisms, by producing bacteriocins or other substances.

Anti mould activity - A mixture of acetic, caproic, formic, propionic, butyric and n-valeric acids, acting in a synergistic way, in which caproic acid played a key role, was responsible for the in vitro inhibitory activity of Lb. sanfranciscensis CB1 against moulds responsible for bread spoilage such as Fusarium, Penicillium, Aspergillus and Monilia (Corsetti *et al.,* 1998).

2.3 *Lactococcus lactis* in enrichment of ɣ-aminobutyric acid in sourdough bread

Fried sourdough bread (bhatura) with an elevated amount of g-aminobutyric acid (GABA) was produced using lactic acid bacteria (LAB). The LAB starter was screened and isolated from pickled yam showing highest GABA content and was identified as Lactococcus lactis subsp. lactis. The maximum GABA production in de Man Rogosa Sharpe (MRS) media supplemented with monosodium glutamate (MSG) was 110 mg/100 ml at pH 5, and 1–3% NaCl did not change the production of GABA significantly ($p < 0.05$). When MSG was replaced with Vigna mungo in sourdough, the amount of GABA for bhatura was 226.22 mg/100 g representing about 10-fold increase. A sensory evaluation resulted as the overall general acceptability of bhatura to be 4.91 ± 0.03 on a five-point hedonic scale. Thus, the results indicated the potential of L. lactis as a LAB starter for the production of GABA-enriched bhatura (Bhanwar *et al.,* 2012). Earlier reports suggest that GABA-enriched foods have been effective in the regulation of sleeplessness, depression and autonomic disorders (Okada *et al.,* 2000).

LAB contribute to the microbial safety or offer one or more organoleptic, technological, nutritional or health advantages, for example, some LAB produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins or useful enzymes, or that have probiotic properties. Acid-producing ability might be an important factor and it does not decrease in processing the food quality and taste of fermented foods. To develop fermented foods containing GABA with good taste, LAB strains with suitable acid and flavour production profiles should be chosen. Because of the high GABA-producing ability, *L. lactis* has great potential for use as a starter in the production of GABA-containing functional fermented foods.

2.4 Microbial degradation of tannins

Tannins are water-soluble polyphenolic compounds having wide prevalence in plants. The microbial degradation of condensed tannins is, however, less than hydrolysable tannins in both aerobic and anaerobic environments. A number of microbes have also been isolated from the gastrointestinal tract of animals, which have the ability to break tannin-protein complexes and degrade tannins, especially hydrolysable tannins. Tannase, a key enzyme in the degradation of hydrolysable tannins, is present in a diverse group of microorganisms, including rumen bacteria. It is well known that tannins are toxic and bacteriostatic compounds making non-reversible reactions with protein (Scalbert, 1991). The production of extracellular tannase (tannin acyl hydrolase; 3.1.1.20) by bacterial cultures with simultaneous release of gallic acid and glucose, was reported for the first time by Deschamps *et al.,* 1983.

2.5 Mechanism of tannin degradation

The gallic acid monomers are readily utilised as substrates by oxidative breakdown to simple aliphatic acids, which then enter the citric acid cycle (Field & Lettinga, 1992b). Prior to ring cleavage, gallic acid is converted to pyrogallol by gallate decarboxylase. The anaerobic decomposition of gallic acid, the monomer of hydrolysable tannins, occurs by different mechanisms. The first step is decarboxylation of gallic acid to form pyrogallol which is then isomerized to phloroglucinol by pyrogallol-phloroglucinol isomerase (Krumholz & Bryant 1988), and to dihydrophloroglucinol by phloroglucinol reductase (Brune & Schink 1990; Brune & Schink 1992). Dihydrophloroglucinol is then converted to 3-hydroxy-5- oxohexanoate

(HOHN) by dihydrophloroglucinol hydrolase. HOHN is degraded by different pathways in anaerobic and in ruminal systems. In the anaerobic system, it is converted to 3, 5-dioxohexanoate (triacetate) by HOHN dehydrogenase and ultimately to three molecules of acetyl-CoA via triacetyl-CoA by the sequential enzymatic action of triacetyl-CoA transferase, triacetate _-ketothiolase, acetoacetyl-CoA _-ketothiolase, phosphotransacetylase and acetate kinase (Brune & Schink 1992).

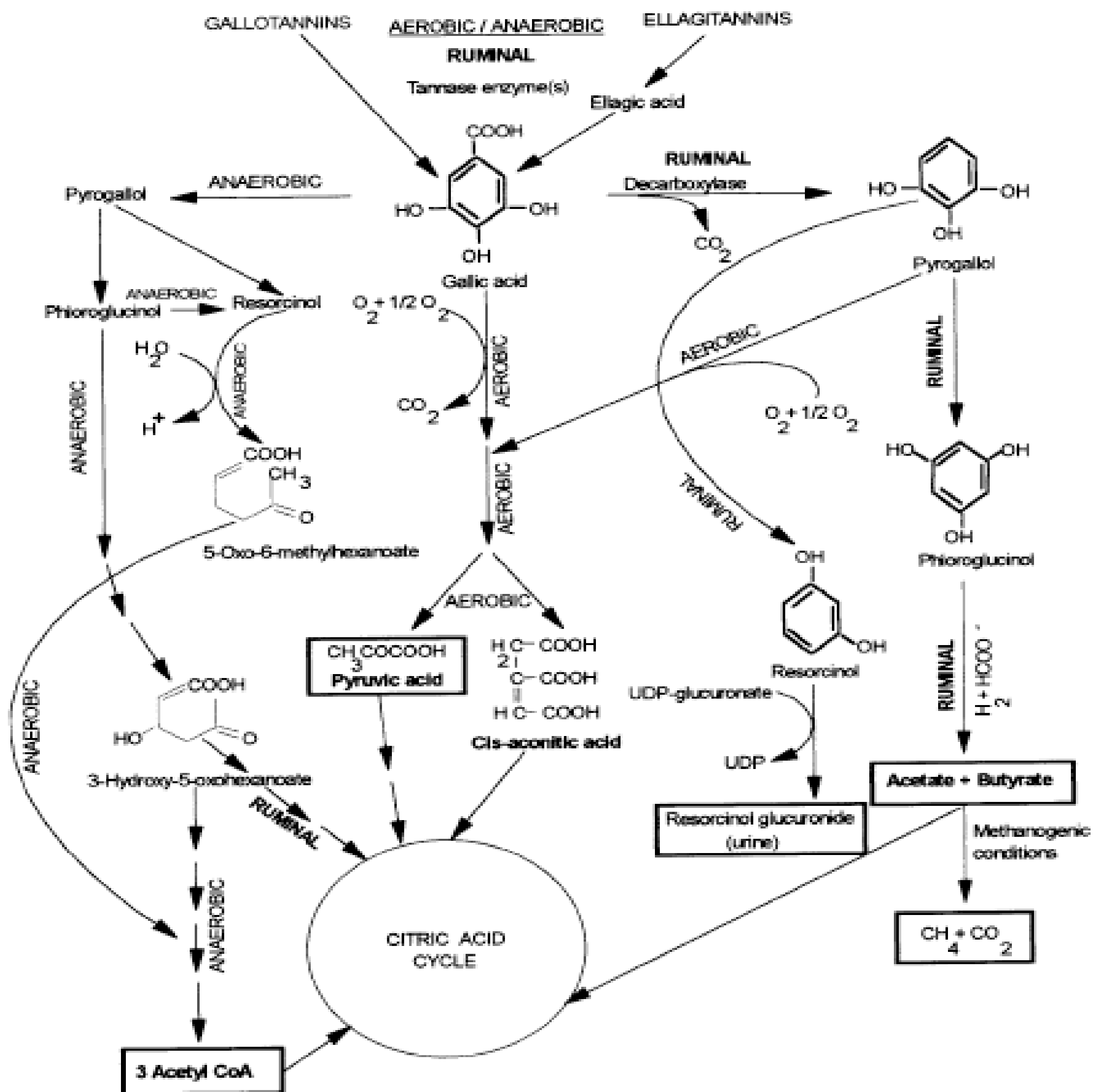

Fig.2.2 Mechanism of tannin degradation (Field & Lettinga, 1992)

Tannins and their interaction with gastrointestinal tract microflora

Tannins inhibit the activity of enzymes of rumen microbes (Mc leod, 1974; Makkar *et al.,* 1998). Condensed tannins from *Lotus corniculatus* have been shown to inhibit extracellular endoglucanase activity of *Fibrobacter succinogenes* (Bae *et al.,* 1993).

For grazing herbivores, tannin present in plants can, in general, adversely affect their nutrition by reducing intake, protein digestibility, inhibiting digestive enzymes or by direct systemic toxicity (Kumar and Singh, 1984). This leads to a reduction in their feed intake, adversely affects rumen fermentation and significantly depresses digestibility of almost all the nutrients. Hydrolysable tannins are toxic and cause poisioning in animals if sufficiently large amounts of tannin-containing plant material, such as leaves of oak and yellow wood (*Terminalia oblongata*) are consumed. They are apparently metabolized by the ruminal microflora to phenolic compounds such as gallic acid, which is neither hepatoxic nor nephrotoxic to animals. However, pyrogallol the decarboxylated product of gallic acid, is produced in high concentration in the rumen of sheep and causes methaemoglobinaemia in the animal (Zhu *et al.,* 1995).

Normally, rumen microbes have capability of degrading and detoxifying many incriminating and antinutritional factors into simpler and non-toxic constituents (Selinger *et al.,* 1996). Some herbivores have developed mechanisms for overcoming adverse effects of tannins. One of the mechanisms may be the harbouring of gastrointestinal microflora which have developed the ability to degrade tannins to innocuous compounds, utilize them for their growth, and may thus contribute to the overall growth of the animal.

2.6 Biotechnological production of Tannase

The enzyme tannin acyl hydrolase (TAH), commonly referred as tannase (EC. 3.1.1.20) is involved in biodegradation of tannins and has important applications in various industries, particularly in food and pharmaceutical sectors. TAH catalyzes the hydrolysis of ester bonds present in gallotannins, ellagitannins, complex tannins, and gallic acid esters. Tannins are resistant to biodegradation, and the accumulation due to discharge of tanneries and coffee
processing industries effluents can result in environmental pollution (Field and Lettinga 1992; Bhat *et al,.* 1998). TAH can be extracted from microbial, animal, and vegetal sources, but

microorganisms are commonly used for commercial production since they produce tannases more stable than the vegetal or animal enzymes. In addition a constant large-scale production of tannase can be achieved by microbial fermentation process (Lekha and Lonsane, 1997). Although it is well known that tannins inhibit microbial growth, there are a number of microbes able to degrade these compounds and even grow with tannic acid as sole carbon source (Banerjee and Pati, 2007). These microorganisms have developed the necessary mechanisms to overcome the inhibitory effect of tannin, such as the production of tannase.

Metabolic regulation of tannase production

Depending on the strain and fermentation conditions, the tannase can be produced either constitutively or by substrate induction. Knudson (1913) reported that tannase production only occurs in the presence of tannic acid, resulting in the formation of gallic acid and glucose as final products. Later, Seiji *et al.* (1973) observed tannase production when the microorganism was grown on glucose as sole carbon source. More recently, Bradoo *et al.* (1997) showed that *Aspergillus japonicus* produced tannase constitutively when grown in a simple culture medium with simple or complex sugars, but the production of the enzyme was doubled when grew up with tannic acid as sole carbon source. The regulation mechanism of TAH is still unclear, and there exist some controversies about the specific role of some compounds in the induction and repression of its expression. It is generally accepted that tannic acid cannot act directly as an inducer; the molecule is very large and reactive to penetrate the cell membrane of microorganisms. Gallic acid, which has been used as inducer for the production of tannase (Bajpai and Patil 1997), has also been linked to its regulation (Bradoo *et al.,* 1997). This suggests that the production of TAH is induced by intermediate compounds produced during the hydrolysis of tannins due to the action of constitutively produced tannase.

Mechanism of tannase action

Although the tannins are known as protein precipitants, the tannase acts over these compounds hydrolyzing the ester bonds formed between galloyl groups and a polyhydroxy alcohol or the depside link between two galloyl groups (Aguilar and Gutierrez, 2001). It has been proposed that the tannase has a depsidase and esterase activity, and this specificity depends on the culture

conditions (Farias *et al.,* 1994). TAH catalyzes the complete hydrolysis of tannic acid to gallic acid and glucose. The intermediates in the reaction are 1,2,3,4,6-pentagalloylglucose, 2,3,4,6-tetragalloylglucose, and two types of mono-galloyl glucose (Iibuchi *et al.,* 1972).

Tannase application and potential uses

Tannase is widely used in the leather, pharmaceutical, beverage, and food industries. So far, the main applications of the tannase are instant tea, acorn liquor, as well gallic acid production from plant materials high in gallotannins. TAH is also used as clarifying agent in juices and flavored coffee soft drinks (Aguilar and Gutierrez-Sanchez, 2001). Gallic acid is used in the pharmaceutical industry as an important intermediate compound in the synthesis of trimethoprim. It is used in the chemical industry as a substrate for chemical or enzymatic synthesis of propyl gallate and other antioxidant compounds, cosmetics, hair products, adhesives, and lubricants. Gallic acid is used also in fabrication of semiconductors, dyes, and in photographic revelation. Several studies have found that gallic acid and related compounds have important therapeutic properties (Abdelwahed *et al.,* 2007). Tannase helps to reduce the adverse effects of tannins in beverages and foods. In the manufacture of instant tea, TAH is used to remove insoluble precipitates that are formed when the drink is cooled to temperatures below 4°C. These precipitates are formed by polymerization of phenolic compounds and their interaction with caffeine.

Rout and Banerjee (2006) reported that tannase treatment reduced tannin content of pomegranate juice by 25%, while the combination of tannase and gelatin removed 49% of the tannins present. The varieties of sorghum with high tannin content are not suitable for animal consumption and treatment with tannase or a tannase-producing microorganism can be implemented to reduce the concentration of anti-nutritional factors and make it a useful food supplement for the animal (Aguilar and Gutierrez, 2001).

CHAPTER 3
MATERIALS AND METHODS

This section of thesis deals with the details of the various experiments which have been carried out.

All the chemicals/Bio chemicals used were of higher analytical grade/purity and purchased from Sigma Aldrich (St. Louis, MO, USA) and HiMedia (Mumbai, India) unless otherwise specified. During this study the chemicals/materials were used for the maintenance of culture, tannin reduction and tannase production.

3.1 Strains and growth conditions

An initial characterization of LAB culture(s) in our laboratory for potential tannin degraders revealed *Lactococcus lactis* strain LAHKT 2 as a suitable tannin degrader. This strain was selected based on its high tannase activity. The strain was routinely grown in DeMan Rogosa and Sharpe agar (MRS) at 37˚C (Bhanwar *et al.,* 2012). Inoculum was prepared by growing the microorganism in 100 ml MRS medium in 250 ml Erlenmeyer flask for 24 hours at 37˚C on a rotary shaker at 120 rpm. All the medium components used in study were purchased from Himedia Ltd., Mumbai, India. Purity was confirmed periodically by gram staining and microscopic observation.

For preparing sourdough, highest grade wheat flour (Aashirvaad select, ITC) and *Vigna Mungo* (flour, graded quality) were used. Tannin, saponin, gallic acid were procured from Sigma (Mo, USA), all other chemicals, reagents were of highest analytical grade.

3.2 Tolerance to tannin

Survival study of bacteria was carried out on MRS agar plates supplemented with different concentrations of filter sterilized tannin (0.08 mM, 0.10 mM, 0.15 mM, 0.2 mM, 0.25 mM, 1 mM, 5 mM, 20mM) and culture viability recorded as log (CFU/ml) for each concentration.

3.3 Reduction of tannin by whole cells and resting cells

Tannin estimation was done as described by (Makkar *et al.,* 1993). Briefly, MRS medium was incorporated with different concentrations of filter sterilized tannin (0.05-0.75 mM). The medium was inoculated with 1% (v/v) of seed culture in the mid exponential phase. After 24 hours of incubation period at 37˚C and pH 6.8 samples were tested for decrease in tannin. 50 micro litre of tannin extract for each sample was taken in a test tube and volume was made to 1.0 ml with distilled water. Then 0.5 ml Folin-Ciocalteu reagent (1N) was added and mixed properly. Then 2.5 ml 20% sodium carbonate solution was added and mixed properly and kept for 40 minutes at room temperature. Optical density was recorded at 725 nm in spectrophotometer and concentration was estimated from the standard curve.

3.4 Growth kinetics of *Lactococcus lactis* in presence and absence of tannin

The tannin concentration at which maximum reduction was obtained was considered for the growth kinetic study with time. A comparative growth kinetic study of *Lactococcus lactis* in presence and absence of tannin was performed by incorporating 0.08 mM filter sterilized tannic acid (i.e. 8 ml of tannic acid /92 ml of MRS medium) in MRS medium, inoculated with 1% (v/v) of culture in log phase. Aliquot of sample from the fermentation broth was withdrawn at 2 hour interval and growth of *L. lactis* was analyzed by reading the absorbance at 600 nm in a spectrophotometer (Hitachi, Japan).

Growth kinetic study of *L. lactis* was also conducted in M17 minimal media and in the medium when tannin was provided as the sole carbon source. The composition of the medium when tannin was provided as the sole carbon source for *L. lactis* was the following: Peptone from casein (10 g/l), Beef extract (10 g/l), Yeast extract (5 g/l), tannic acid (0.08 mM), dipotassium hydrogen phosphate (2 g/l), Tween 80 (1g/l), triammonium citrate (2 g/l), sodium acetate (5 g/l), magnesium sulphate (0.2 g/l), manganese sulphate (0.04 g/l) and reading was taken at 600 nm for growth analysis.

3.5 Kinetics of tannin degradation by growing cells and resting cells

The tannin concentration at which maximum reduction was obtained was considered for the kinetic study of degradation of tannin with time. Kinetic study of degradation of tannin was conducted by incorporating 0.08 mM filter sterilized tannic acid (i.e. 8 ml of tannic acid /92 ml of MRS medium) in MRS medium, inoculated with 1% (v/v) of culture in log phase. Aliquot of sample from the fermentation broth was withdrawn at 2 hour interval and tannin degradation was estimated as mentioned in section 3.4.

3.6 Biochemical examination of tannin degradation by *L. lactis* tannase

The tannase production by *L. lactis* was conducted in 250 ml Erlenmeyer flask with 100 ml of the production medium. The pH of the medium was adjusted to 6.8 using 1M NaOH or 1N HCl and sterilized (121˚C for 15 min). The production medium was inoculated with 1% of the seed culture in the log phase and incubated at 37˚C in an incubator for 24 hours. After 24 hours the cells were separated from the fermentation medium by centrifugation at 8000 rpm for 5 minutes. The cell pellets were washed three times with 0.85% saline. The cell pellets were re-suspended in 20 ml saline for cell rupture. The cell disruption steps were carried out in ice to ensure low temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 12,000g for 20 min at 4 ˚C and supernatant and pellet were separated. Clarified supernatant was obtained and these were further precipitated with ammonium sulphate and partially purified through dialysis to obtain partial purified extracellular and intracellular tannase which were further used for analysis of tannase activity. The tannase activity was estimated by the procedure of (Deschamps *et al.,* 1983) and enzyme activity was measured in terms of amount of gallic acid produced.

3.6.1 Analysis of tannase activity; PMSF treatment and thermostablity

Partially purified tannase was treated with phenyl methyl sulfonyl fluoride (PMSF) to check for the activity of intracellular and extracellular tannase. 17.4 mg PMSF was added to 10 ml of isopropanol and stored at -20˚C for further use. 1 ml PMSF was added to 2 ml of each partially purified samples and further test for the activity by the procedure of (Deschamps *et al.,*1983).

Extracellular and intracellular enzyme was also tested for its thermal stability by heating the sample at a temperature of 50˚C, 80˚C and 100˚C and then activity analysis was done by the same procedure.

3.6.2 Tannase detection : Zymography

Polyacrylamide gel was prepared using the method of (Laemmli, 1970). Separating gel (10%) was pre- lectrophoresed at 2 mA for 2 h to remove the residual ammonium persulphate and TEMED. After that 5% stacking gel was poured. The enzyme was loaded and electrophoresed at 10 mA for 2 h under cold environment. The gel was then dipped into 0.5% w/v tannic acid solution (in 0.2 M acetate buffer, pH 6.0) for 30 min at room temperature. After that, the gel was rinsed with the same buffer for several times and flooded with BSA solution [0.17 M sodium chloride and BSA 2 mg/ml (Sigma, USA) in 0.2 M acetate buffer (pH 5.0)] and incubated for 15 min at 4˚C for tannic acid–BSA complex formation.

3.6.3 Estimation of total extracellular and intracellular protein

The amount of secretion of total extracellular and intracellular protein by *L. lactis* in presence of tannin was estimated by the procedure of (Bradford, 1976). *L. lactis* was grown MRS media in presence of 0.08 mM tannic acid at a temperature of 37˚C and pH 6.8. After 24 hours of incubation the sample was centrifuged at 8000 rpm for 10 min at 4˚C and pellet and supernatant were separated. The pellet was washed three times with 0.85% saline and then resuspended in 10 ml saline for cell rupture. Cells were ruptured using bead beater and then centrifuged at 12000*g for 20 min at 4˚C to obtain clear supernatant. Both the supernatant obtained were used for protein estimation.

3.7 Optimization of kinetics of tannin degradation

The effect of different temperatures, pH, nitrogen source and inoculum size on tannin degradation with time was studied. Tannase activity was assayed by estimating tannic acid reduction as mentioned in section 3.4.

3.7.1 Optimum temperature and pH for tannin degradation

To determine the optimal pH for tannin degradation, samples were adjusted at different pH values (5-8) using 1M NaOH and 1N HCl. For optimum temperature determination the samples were placed at varying temperature conditions (28˚C, 37˚C and 42˚C) and aliquot of samples from fermentation broth was withdrawn every 2 hours and tannin estimation was performed as described in section 3.4.

3.7.2 Effect of varying nitrogen source, inoculums size and agitation on tannin degradation

Samples were prepared by removing various nitrogen sources keeping one as constant. In MRS media there are three sources for nitrogen, peptone, beef extract and Yeast extract. Three different samples were prepared by removing one of the nitrogen sources from MRS media keeping others as constant and tannin estimation was done for each sample at an interval of 2 hours. The effect of inoculums size on tannase activity was done by inoculating with different inoculums size of 1%, 2% and 3%. Effect of agitation on tannase production and respective tannin degradation was examined by incubation of samples on a rotary shaker at 80, 100 and 120 rpm and without agitation.

3.8 Role of *L. lactis* biofilm in tannin degradation

Three wells of two rows of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of bacterial suspension each. To the wells of one row 0.08 mM tannin was also added. Negative control wells contained broth only. The plates were covered and incubated aerobically for 72 h at 37°C. Then, the content of each well was aspirated, and each well was washed three times with 250 µl of sterile physiological saline. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200 µl of 99% methanol per well, and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 0.2 ml of 2% Hucker crystal violet used for Gram staining per well. Excess stain was rinsed off by washing the wells with deionized water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using an automated High throughput reader. The reading was performed two times: (i) before addition

of glacial acetic acid, as in standard microtiter-plate test and (ii) after glacial acetic acid was added.

The amount of tannin degraded by the bacterial cells after biofilm formation was also calculated by the procedure as described in section 3.4 and comparison of growth *L. lactis* cells in planktonic form and in biofilm form was done by taking the dry weight of both the planktonic and biofilm formed cells.

3.9 Tannin estimation in composite sourdough batter

A composite sourdough batter using wheat flour and *vigna mungo* flour was prepared according to the procedure of (Bhanwar *et al.,* 2013). 100 g flour of each *vigna mungo* flour and wheat flour was weighed. To it 1% *L. lactis* inoculums was added and the batter was prepared by adding water to it. Two samples were taken, one inoculated with 1% seed culture and other sample without seed culture and tannin estimation was carried out for both the samples of composite sourdough after 4 hours of fermentation at 37˚C.

3.9.1 Estimation of carbohydrates, proteins, vitamin C, Riboflavin in composite sourdough batter

Total amount of carbohydrates present in composite sourdough was determined by the method of (Dubois *et al.,* 1956) and vitamin C estimation was done by the method of redox titration using iodine solution (British Pharmacopeia 2003; Page 163, 164). The reaction proceeded with the oxidation of ascorbic acid by iodine to dehydroascorbic acid and conversion of iodine to iodide. Once all the ascorbic acid has been oxidized, the excess iodine is free to react with the starch indicator, forming the blue-black starch-iodine complex. This is the endpoint of the titration. To 10 ml aliquots of food extracts in 250 ml conical flasks, 4-5 drops of 1 % starch indicator was added. Titration was performed with 0.05 M iodine solution till the end point (blue-black colour, due to starch-iodine complex) was reached. The concentration of ascorbic acid present in the food extract was estimated from a standard plot where L–ascorbic acid was used in varying concentrations.

Estimation of riboflavin was determined colorimetrically, 0.8 ml of the sample was added to 0.2 ml of 1 M Sodium Hydroxide. To 0.4 ml of the resulting solution, 1 ml of 0.1 M potassium phosphate buffer (pH 6) was added to neutralize. Absorbance at 444 nm was measured using a spectrophotometer. Concentration of riboflavin in the food samples was estimated with riboflavin as standard.

CHAPTER 4

Results and discussions

4.1 Survival of *L. lactis* / tannin tolerance

The survival of the cultures following incubation with different concentrations of tannic acid (Figure 4.1) indicated that *L. lactis* can best tolerate a concentration of 0.08 mM of tannin while no growth occurred upon incubation with 20 mM of tannic acid. Survival study further indicated that *L. lactis* can grow better in low tannin concentration while it is not able to tolerate high conc. of tannin and it was in accordance with the work of (Singh *et al.,* 2011), where they reported maximum tolerance of 20 mM.

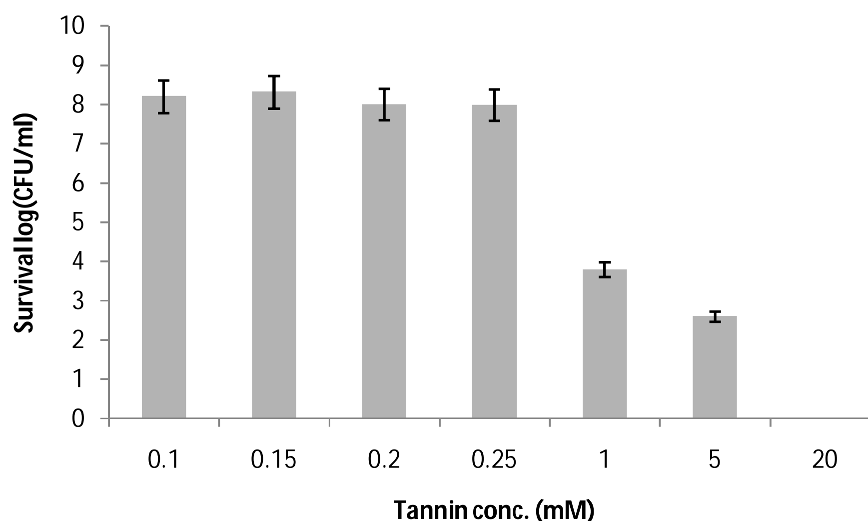

Figure 4.1. Tolerance to tannin by *L. lactis* in MRS medium

4.2 Reduction of tannin by whole cells

Tannin reduction results revealed (Figure 4.2), maximum reduction at a conc. of 0.08 mM. The tannin's phenolic group is an excellent hydrogen donor that forms strong hydrogen bonds with the protein's carboxyl group. For this reason, tannins have a greater affinity to proteins than to starch. Soluble complexes are favored when protein concentration is in excess (fewer tannin

attachment sites per each protein molecule) while Insoluble complexes are formed when tannins are present in excess and form an hydrophobic outer layer in the complex surface and it is expected that at higher tannin concentration large amount of proteins gets precipitated and hence, bacteria is able to tolerate less concentration of tannin. Kinetics of tannin degradation by *L. lactis* suggests a maximum reduction of tannin after 9 hours of incubation period with a sharp decrease after 16 hours of incubation (Figure 4.4).

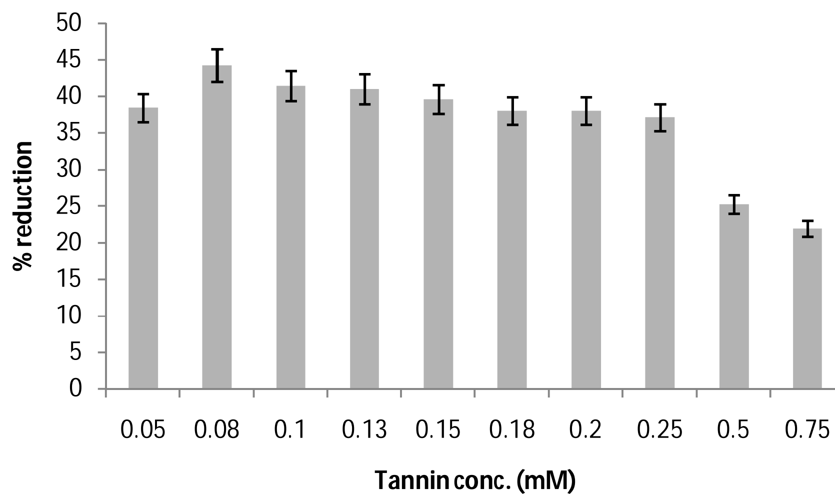

Figure 4.2 Reduction of tannin by *L. lactis* whole cells

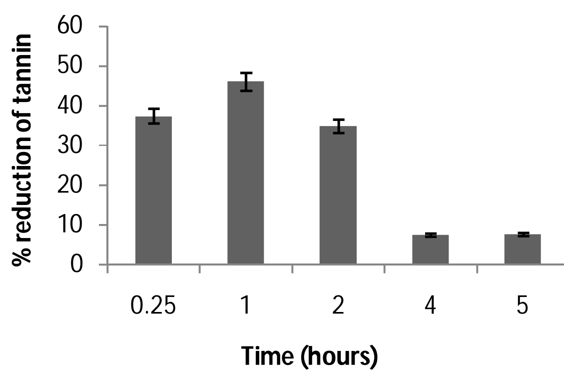

Figure 4.3 Reduction of tannin by resting cells of *L. lactis*

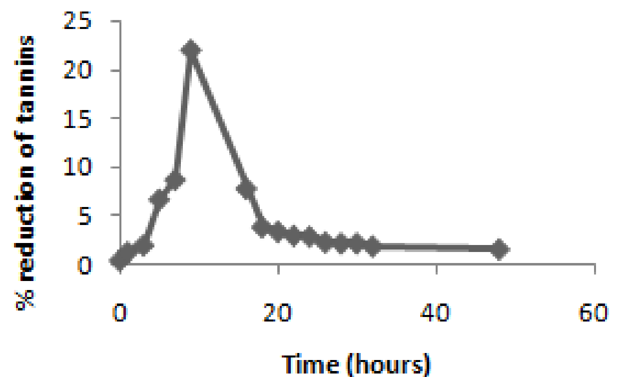

Figure 4.4 Kinetics of tannin degradation by *L. lactis*

4.3 Growth kinetics of *Lactococcus lactis* in presence and absence of tannin

A correlation with the growth kinetics of *L. lactis* (Figure 4.5) indicated the cells to be at early exponential phase at which maximum degradation of tannin is observed; hence maximum tannin was utilized to produce gallic acid and glucose which is required by the bacteria for its growth. It was observed that when allowed to grow in presence of tannin the cells were in lag phase till 5th hour while without tannin it was till 2 hours. An extended log phase observed upon addition of tannin may be experienced by the fact that tannin bind to protein in MRS media making it unavailable fot the culture to grow. The specific growth for *L. lactis* in presence of tannin was found to be $0.11h^{-1}$ while in absence of tannin it was $0.48\ h^{-1.}$ Tannase production & tannin degradation was dependent on growth and resting cells could hardly degrade tannin. Growth kinetics of *L. lactis* when tannic acid was provided as the sole carbon source (Figure 4.6) shows the maximum growth at 19 hours with a specific growth rate of $0.13\ h^{-1}$ while maximum growth when grown in M17 minimal media (Figure 4.7) was 24 hours with a specific growth rate of $0.49\ h^{-1}$, showing clearly that *L. lactis* utilizes tannin as a carbon source when it was allowed to grow in MRS media in presence of tannin and maximum growth was obtained after 26 hours of incubation while in absence of tannin at 24 hours, this shows that there is no any adverse effect of tannin on the growth of *L. lactis*.

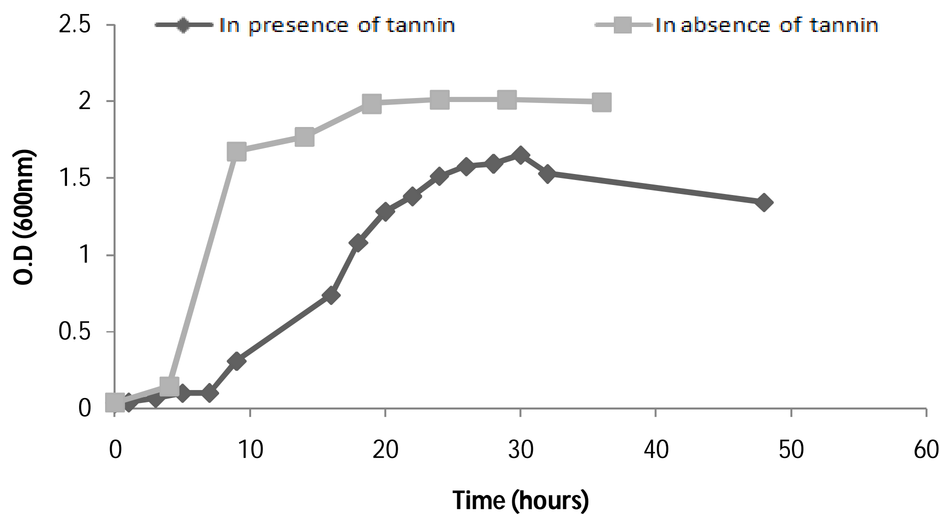

Figure 4.5 Growth kinetics of *L.lactis* in presence and absence of tannin

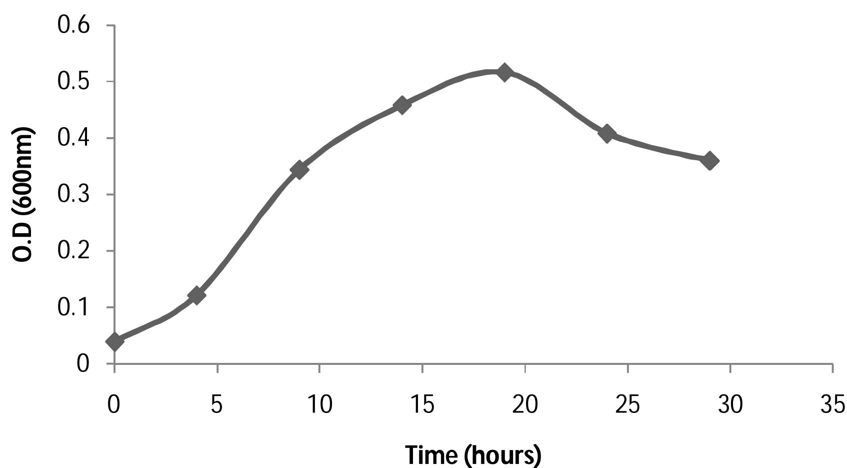

Figure 4.6. Growth kinetics of *L. lactis* when tannin was provided as a sole carbon source

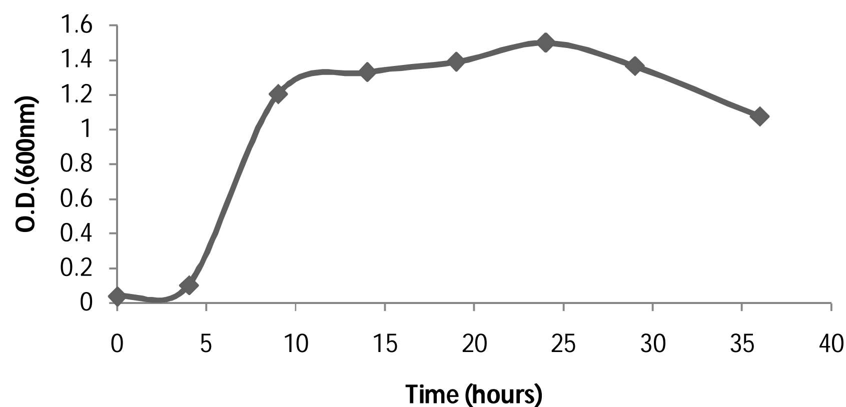

Figure 4.7 Growth kinetics of *L. lactis* in M17 minimal medium

4.4 Tannase production

Tannase catalyzes the hydrolysis of ester linkages of the tannic acid producing gallic acid and thus the tannase activity could be measured by gallic acid estimation. A method proposed by (Deschamps *et al.,* 1983) was used for quantification of tannase activity where absorbance was read at 260 nm and amount of gallic acid produced in the reaction mixture was estimated from the standard curve of gallic acid. The acceptable daily intake (ADI) of gallic acid as described by

(Heijdin *et al.,* 1986*)* is 0.2mg/kg body weight. The enzyme production was inducible in nature and was found to be produced both intracellularly and extracellularly (Figure 4.8, 4.9) while we tested for the activity of both extracellular and intracellular tannase. Addition of PMSF can lead to proteolytic inhibition when a concentration of 0.1-1 mM PMSF is used. PMSF specifically binds to the active site serine residue in a serine protease, but does not bind to any other serine residues in the protein.

Therefore addition of PMSF can inactivate enzyme activity and thus tannase is inactivated, reduction of tannin will not be observed in the supernatant containing extracellular and intracellular tannase, almost no reduction in tannin concentration with time was observed as anticipated. When the sample containing both extracellular and intracellular tannase was heated at 100 ˚C for 1 hour 30 minutes, no alteration in enzyme activity was obtained and this proved it to be thermostable in nature. The tannase activity was analysed by the amount of tannin degradation or by the mount of gallic acid produced.

The observation is surprising as thermostable tannase in *L. lactis* has not yet been reported. The result needs further investigation as thermostability confers several practical advantages to enzymes.

Table 4.1 Estimation of tannase activity

Time (hours)	**Intracellular tannase +Tannic acid+PMSF**	**Intracellular tannase (heated @100˚c for 1h 30 min.+Tannic acid**	**Intracellular Tannase + Tannic acid**	**Extracellular Tannase +Tannic acid +PMSF**	**Extracellular tannase (heated @100˚c for 1h 30 min.) +Tannic acid**	**Extracellular tannase + Tannic acid**
Tannin conc. (mM) after **1hour**	**0.079±0.06**	**0.011±0.02**	**0.013± 0.01**	**0.072±0.05**	**Not detected**	**Not detected**
Tannin conc. (mM) after **3 hour**	**0.078±0.05**	**Not detected**	**Not detected**	**0.0650.06**	**Not detected**	**Not detected**

Table 4.2 Gallic acid production by tannase

	Intracellular tannase (heated @100˚c for 1h 30 min.+Tannic acid	Intracellular Tannase + Tannic acid	Extracellular tannase (heated @100˚c for 1h 30 min.) +Tannic acid	Extracellular tannase + Tannic acid
Gallic acid conc. (mM) after **1hour**	**5.09±0.68**	**3.89±0.57**	**3.76±0.61**	**4.74±0.49**
Gallic acid conc. (mM) after **3 hour**	**3.65±0.39**	**3.4±0.43**	**3.04±0.33**	**4.01±0.42**

4.5 Native gel method for tannase detection

The tannase within the gel was specifically hydrolysed tannic acid and thereby formed a clear and transparent zone in the gel. Tannin–protein interactions that give rise to a white coloration in the surrounding gel are based on hydrophobic and hydrogen bonding. Therefore the transparent zone indicated the presence of tannase. This method is the most uncomplicated procedure for tannase visualization (Maity *et al.*, 2009).

Figure 4.8 Zymography of tannase

4.6 Optimization of kinetics of tannin degradation

The effect of varying conditions on *L. lactis* tannase was estimated by the procedure of (Makkar *et al*., 1993). Tannase activity was measured by the reduction of tannin; hence, more the tannin reduction, higher is the tannase activity. The optimum temperature, pH and inoculums size for tannase activity was found to be 37˚C, 6.8 and 1% inoculum respectively (Figure 4.9, 4.10, 4.11). Tannase activity was notably influenced by increase or decrease from optimal pH and agitation also affected tannase activity. Notable decrease in tannase activity occurred when culture was agitated at 120 rpm.

Nitrogen source affected the tannase activity – absence of beef extract resulted in maximal tannin reduction (37%) followed by removal of peptone which showed a reduction of (16.7%) after 2 hours of incubation (Table 1).

The above results were in concurrence to those suggested by other workers, barring that of pH, where our results indicated maximal activity at pH 6.8. The tannase activity remained unaffected at higher temperature indicating it to be thermostable.

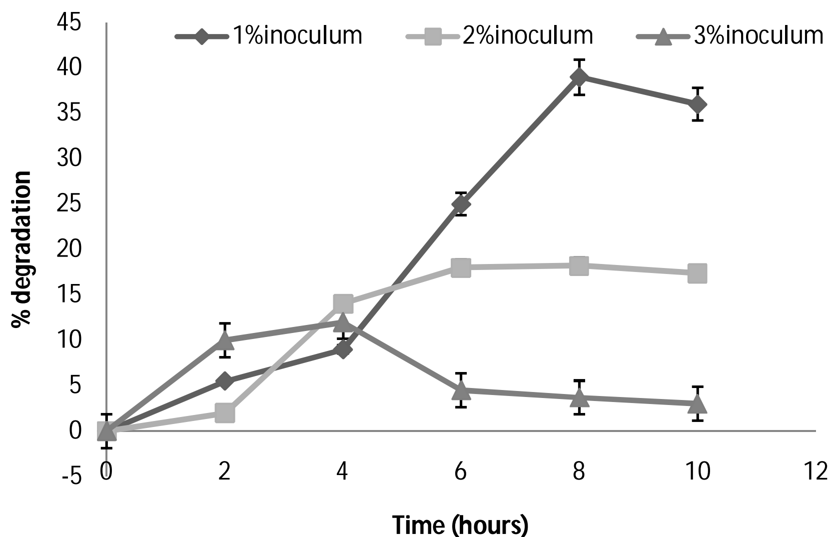

Figure 4.9 Effect of inoculums size on tannin degradation

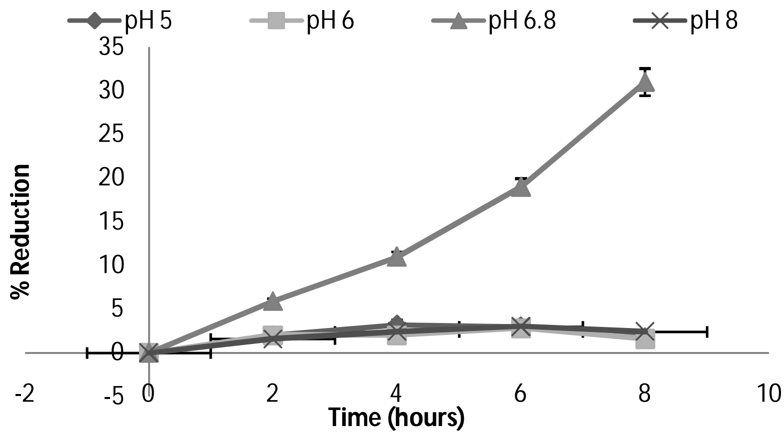

Figure 4.10 Effect of pH on tannin degradation

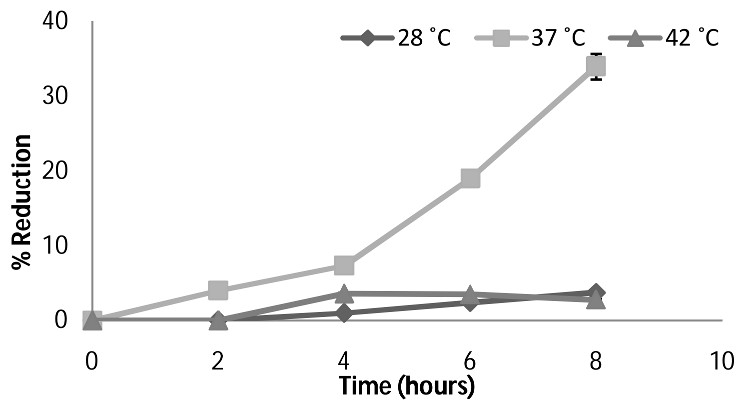

Figure 4.11 Effect of temperature on tannin degradation

Table 4.4 Effect of agitation and varying nitrogen source on tannin degradation

	% reduction at 0 hour incubation	% reduction after 2 hour incubation	% reduction after 4 hour incubation	% reduction after 6 hour incubation
MRS without Beef extract	0	37	4	2
MRS without Peptone	0	16.7	4.08	2.16
MRS without Yeast extract	0	2.6	6.5	4
Agitation 120 rpm	0	2	4	7
No agitation	0	6.7	26.4	35.6

4.7 *L. lactis* biofilm formation

Biofilms is a communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community. The results suggest that there is more biofilm development in presence of tannin in comparison to planktonic cells.

The comparison of the dry weight of planktonic cells and the dry weight of cells forming biofilm also indicated the development of biofilm was preferred. The results are important to indicate that for *L. lactis*, the presence of biofilm is not affected as biofilms are important in gut and presence of tannin does not render *L. lactis* in gut effective of colonization.

Table 4.5 Biofilm development in presence and absence of tannin

Biofilm development in presence of tannin	1.672
Biofilm development in absence of tannin	1.244
Dry weight of planktonic cells	19 mg/ ml
Dry weight of biofilm adhered cells	28 mg/ ml

4.8 Tannin estimation in composite sourdough batter

Complete reduction of tannin (99.98%) was observed following 4 hours of fermentation. Importantly, tannase activity was not affected by the sourdough matrix. Total amount of condensed and hydrolysable tannin present in sourdough before and after fermentation was estimated, which indicated approximately 100 % reduction of hydrolysable tannin and 36.47 % reduction of condensed tannin, thus emphasizing the applicability of *L. lactis* to be a potent strain for reducing antinutritional components at least for tannins.

Table 4.6 Estimation of condensed and hydrolysable tannin in composite sourdough batter

Sample	Tannin concentration (mM) without fermentation (not inoculated with *L. lactis*)	Tannin concentration (mM) after 4 hours fermentation (inoculated with 1% *L. lactis* inoculums)
Sourdough (condensed tannin estimation)	0.788±0.12	0.48±0.09
Sourdough (hydrolysable tannin estimation)	0.038±0.010	0.002±0.001

Table 4.6 shows selected profile of nutritional parameters in composite sourdough. None of these were affected through the process of tannin degradation by *L. lactis*.

Table 4.7 Estimation of protein, carbohydrate, vitamin C and Riboflavin in composite sourdough batter

Sourdough sample	Protein estimation	Vitamin C estimation(mg/ml)	Riboflavin estimation(mM)	Carbohydrate estimation (mg/ml)
Inoculated with *L. lactis*	2.671±0.91	1.1±0.67	0.056±0.003	1191
Not inoculated with *L. lactis*	0.983±0.27	0.067±0.04	0.076±0.021	1109

CONCLUSIONS

The main objective of this study was to develop a nutritionally enriched composite sourdough batter. For this purpose, a high tannase producing strain of Lactic acid bacteria, *L. lactis* subsp *lactis* was evaluated. Since tannin is the principle antinutrient in the composite sourdough.

i) In this study a high tannase producing strain of lactic acid bacteria *L. lactis* subsp *lactis* was evaluated.

ii) *L.lactis* was able to degrade tannin at a low concentration (0.08mM) while it was not able to survive at high concentration.

iii) Tannase production was inducible, thermostable and detected both intracellularly and extracellularly while intracellular showed higher activity.

iv) Optimization of conditions for tannin degradation showed that maximum degradation was obtained at 37°C, 1% inoculums, pH 6.8 and without agitation.

v) Biofilm formation by *L. lactis* was not affected by the presence of tannin.

vi) Adequate viability of *L. lactis* subsp *lactis* in sourdough amended with *Vigna mungo* flour was observed with high tannin reduction.

vii) Tannin degradation by *L. lactis* remained unaffected under sourdough matrix conditions factors namely, phytate and saponin content, temperature and agitation rate.

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APPENDIX I

- **MRS Media Composition (for 250ml):**

Peptone with casein – 2.5gm

Beef extract – 2.5gm

Yeast – 1.25gm

Dextrose – 5gm

Dipottassium hydrogen phosphate – 0.5gm

Tween 80 – 0.25gm

Triammonium citrate – 0.5gm

Sodium acetate – 1.25gm

Magnesium sulphate – 0.05gm

Magnese sulphate – 0.02 gm

- **REAGENTS FOR PROTEIN ESTIMATION:**

Reagent A:2% of sodium carbonate in 0.1N of NaOH

Reagent B:1% of NaK tartarate in water

Reagent C:0.5% of copper sulphate in water.

Reagent 1:

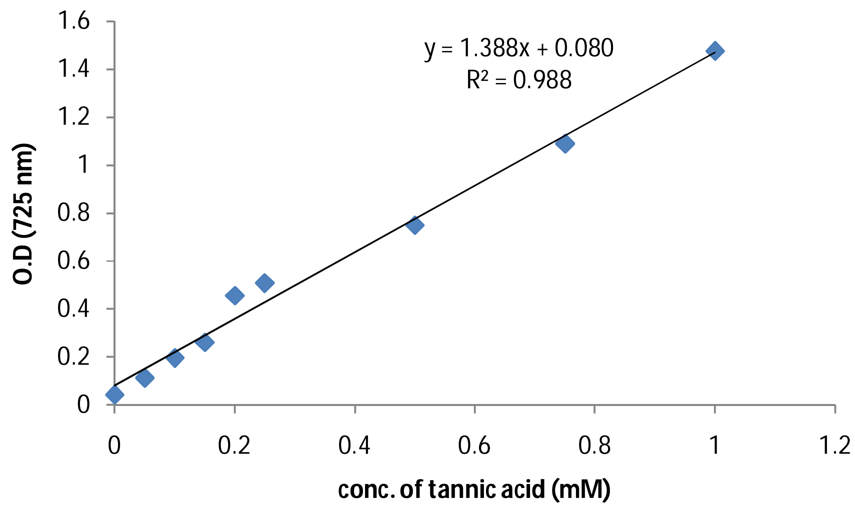
48 ml of reagent A +1ml of regent B+ 1ml of reagent C

Reagent2:

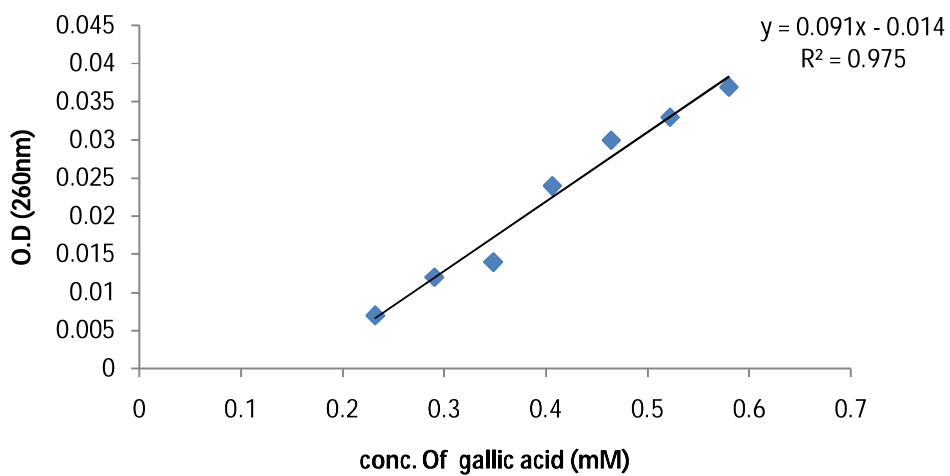
1 part of folin-phenol[2N]:I part of water

BSA standard-1mg/ml

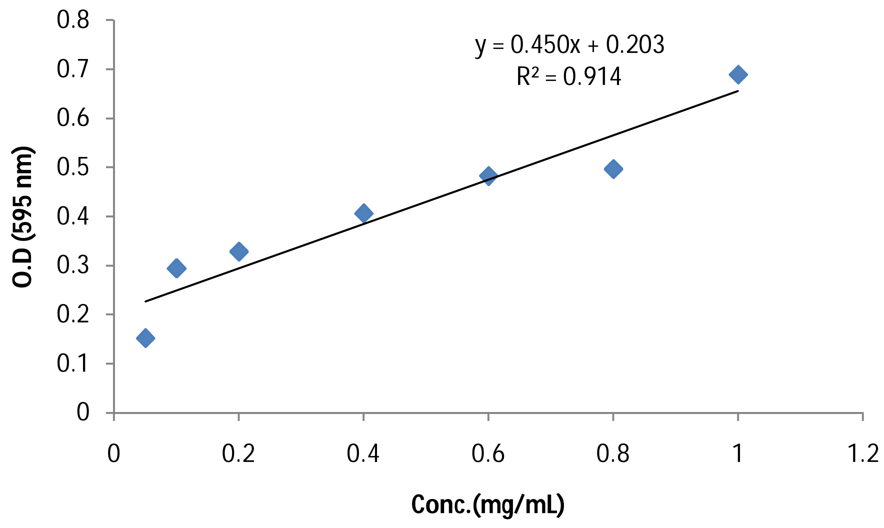
- **Standard curve of tannic acid**



- **Standard curve of Gallic acid**



- **Standard curve of BSA**



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Characterization of tannase production by *Lactococcus lactis* subsp *lactis* and its potential in enhancing nutritional value of a composite sourdough

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Abstract

In the present study we report the tannin degradation ability of probiotic strain *Lactococcus lactis* subsp *lactis*. This strain was able to tolerate tannin concentration of 0.25 mM, tannin degradation occurred primarily by enzyme tannase and significant ($p<0.05$) degradation (44.2%) was observed at tannin concentration of 0.08mM, pH-6.8, temperature 37˚C and without agitation after 6 hours of fermentation in MRS media. Tannase production was inducible, was found to be thermostable and detected both intracellularly and extracellularly. Intracellular tannase showed higher activity in comparison to extracellular, was not affected by the presence of antinutrients such as saponin, phytic acid and lectin. In order to access the applicability of *L. lactis*, an experimental composite sourdough containing *Vigna mungo* and wheat flour was prepared. Fermentation was initiated by inoculating overnight culture of *L. lactis* followed by incubation at 37˚C for 4 hours. Analysis of sourdough extracts indicated a complete reduction of tannin (100%), saponin (72%) and lectins - the principal anti-nutrients in *Vigna mungo*. The result of this study suggests a potential applicability of *L. lactis* subsp *lactis* in removing antinutrients, thus enhancing the nutritional value of traditional Indian sourdough.
Keywords- Antinutrients, Tannin, Saponin, Sourdough, L. lactis

1. Introduction

Tannins are water soluble polyphenolic compound with molecular weight ranging from 0.3-5KDa (Bate-Smith and Swain, 1962) which acts as an antimicrobial agent and occurs primarily in food and feed. Presence of tannin, affects amino acid availability and inhibit activities of various digestive enzymes restricting complete

nutrition. Legumes occupy an indespensible part of regular diet in the Indian subcontinent and is highly important as a major source of proteins; however the presence of antinutrients like tannins, phytic acid, saponins in many legumes hinder their utilization and nutritional importance in mammals. For instance, Saponins through intralumenal physiochemical interaction reduces uptake of nutrients like glucose and cholesterol and hence shows hypocholesterolemic effects (Price *et al.,* 1987) while lectins selectively bind to different types of blood cells and show hemagluttinating ability (Suseelan *et al.,* 1997). In a previous study we developed a composite sourdough-since nutritionally traditional sourdough is of less significance therefore composite sourdough prepared using *Vigna mungo* may improve nutritional value. This is possible since *Vigna mungo* contains about 24% of dietary proteins but it is necessary to reduce antinutritional components like tannins, saponins, phytic acid etc. to enhance the protein digestibility (Bhanwar *et al.,* 2011). In this regard, microbial degradation of tannins in legumes may present a solution for nutritional sustainability and utilization of under-utilized legumes.

However, few microorganisms can degrade tannin and utilize it as nutrient (Lekha and Lonsane, 1997).Tannase is an inducible enzyme which can catalyze the ester and the depside bond of hydrolysable tannins and hydrolyses it completely to gallic acid and glucose. Tannase is an industrially important enzyme with various industrial applications such as food processing, textile and tanneries waste treatment, animal feed production, debittering of fruit juices and gallic acid produced is used in the manufacturing of antimalarial drug. Moreover, microbial tannase are considered to be commercially important on account of their stability and ability to withstand extreme conditions. In the present work, *L. lactis* was investigated for its tannin degradation ability, initially in media. Tannase production was maximized through optimization of culture variables. Finally, the efficacy of tannin degradation (tannase production) by *L. lactis* was evaluated in a composite sourdough containing *Vigna mungo*. The presence of saponin, another principal antinutrient following fermentation with *L. lactis*, is also reported.

2. Materials and Methods

2.1 Microorganism and culture conditions

An initial characterization of LAB culture(s) in our laboratory for potential tannin degraders revealed *Lactococcus lactis* strain LAHKT 2 as a suitable tannin degrader. This strain was therefore used for the study. The strain was routinely grown in DeMan Rogosa and Sharpe agar (MRS) at 37˚C. Inoculum was prepared by growing the microorganism in 100 ml MRS medium in 250 ml Erlenmeyer flask for 24 hours at 37˚C on a rotary shaker at 120 rpm. All the medium components used in study were purchased from Himedia Ltd., Mumbai, India. Purity was confirmed periodically by gram staining and microscopic observation.

For preparing sourdough, highest grade wheat flour (Aashirvaad select) and *Vigna Mungo* (flour) were used. Tannin, saponin, gallic acid were procured from Sigma (Mo, USA), all other chemicals, reagents were of highest analytical grade.

2.2 Tolerance to tannin

Survival study of bacteria was carried out on MRS agar plates supplemented with different concentrations of filter sterilized tannin (0.08 mM, 0.10 mM, 0.15 mM, 0.2 mM, 0.25 mM, 1 mM, 5 mM, 20mM) and culture viability recorded as log (CFU/ml) for each concentration.

2.3 Tannin reduction by whole cells

Tannin estimation was done as described by (Makkar *et al.,* 1993). Briefly, MRS medium was incorporated with different concentrations of filter sterilized tannin (0.05-0.75 mM). The medium was inoculated with 1% (v/v) of seed culture in the mid exponential phase. After 24 hours of incubation period at 37˚C and pH 6.8 samples were tested for decrease in tannin.

2.4 Kinetics of tannin degradation

The tannin concentration at which maximum reduction was obtained was considered for the kinetic study of degradation of tannin with time. A growth kinetic study of *Lactococcus lactis* in presence of tannin and degradation of tannin was conducted by incorporating 0.08 mM filter sterilized tannic acid (i.e. 8 ml of tannic acid /92 ml of MRS medium) in MRS medium, inoculated with 1% (v/v) of culture in log phase. Aliquot of sample from the fermentation broth was withdrawn at 2 hour interval and tannin degradation was estimated as mentioned in section 2.3, while growth of *L. lactis* was analyzed by reading the absorbance at 600 nm in a spectrophotometer (Hitachi, Japan).

2.5 Production of tannase in media

The tannase production by *L. lactis* was performed in 250 ml Erlenmeyer flask with 100 ml of the production medium. The pH of the medium was adjusted to 6.8 using 1M NaOH or 1N HCl and sterilized (121˚C for 15 min). The production medium was inoculated with 1% of the seed culture in the log phase and incubated at 37˚C in an incubator for 24 hours. After 24 hours the cells were separated from the fermentation medium by centrifugation at 8000 rpm for 5 minutes. Clarified supernatant was obtained which was further used for analysis of tannase activity. The tannase activity was estimated by the procedure of (Deschamps *et al.,* 1983).

2.6 Enzyme characterization

The effect of different temperatures, pH, nitrogen source and inoculum size on tannase activity from *L. lactis* was studied. Tannase activity was assayed by estimating tannic acid reduction as mentioned in section 2.3.

2.6.1 *Optimum temperature and pH for tannase activity*

To determine the optimal pH for tannase activity, samples were adjusted at different pH values (5-8) using 1M NaOH and 1N HCl. For optimum temperature determination the samples were placed at varying temperature conditions (28˚C, 37˚C and 42˚C) and aliquot of samples from fermentation broth was withdrawn every 2

hours and tannin estimation was performed as described in section 2.3.

2.6.2 *Effect of varying nitrogen source, inoculums size and agitation on tannase activity*

Samples were prepared by removing various nitrogen sources keeping others as constant. In MRS media there are three sources for nitrogen, peptone, beef extract and Yeast extract. Three different samples were prepared by removing one of the nitrogen sources from MRS media keeping others as constant and tannin estimation was done for each sample at an interval of 2 hours. The effect of inoculums size on tannase activity was done by inoculating with different inoculums size of 1%, 2% and 3%. Effect of agitation on tannase production was examined by incubation of samples on a rotary shaker at 80, 100 and 120 rpm and without agitation.

2.7 Tannin estimation in composite sourdough

A composite sourdough was prepared according to the procedure of Bhanwar *et al.,*(2013). Two samples were taken, one inoculated with 1% seed culture and other sample without seed culture and tannin estimation was carried out for both the samples of composite sourdough after 4 hours of fermentation at 37˚C.Saponin was estimated as described by Uematsu *et al.* (2000).

3. Results & Discussion

3.1 Survival of L. lactis and tannin reduction

The survival of the cultures following incubation with different concentrations of tannic acid (Figure 1) indicated that *L. lactis* can best tolerate a concentration of 0.08 mM of tannin while no growth occurred upon incubation with 20 mM of tannic acid. Survival study further indicated that *L. lactis* can grow better in low tannin concentration while it is not able to tolerate high conc. of tannin. Tannin reduction results revealed (Figure 2), maximum reduction at a conc. of 0.08 mM.

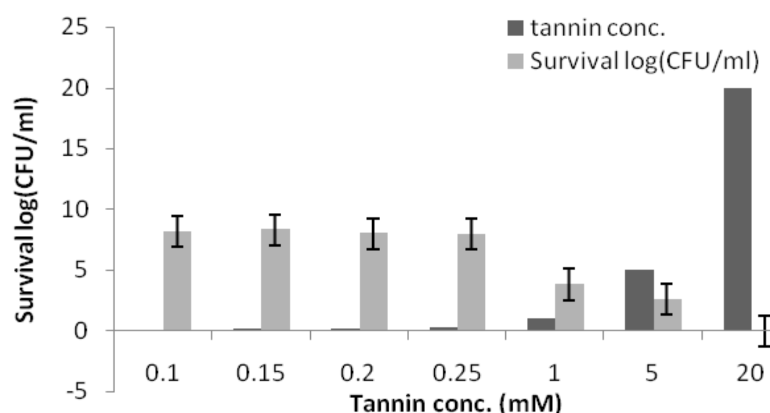

Figure 1. Tolerance to tannin

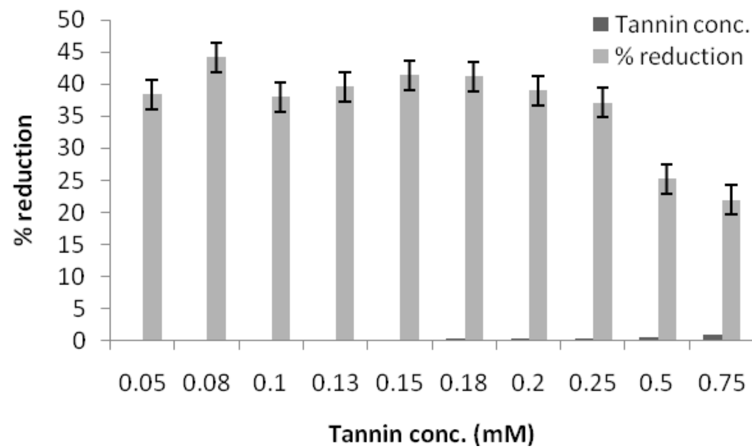

Figure 2. Reduction of tannin by whole cells

3.2 Kinetics of tannin degradation and L. lactis growth

Tannic acid degradation by *L. lactis* suggests a maximum reduction of tannin after 9 hours of incubation period with a sharp decrease after 16 hours of incubation (Figure 3). A correlation with the growth kinetics of *L. lactis* (Figure 4) indicated the cells to be at early exponential phase; hence maximum tannin was utilized to produce gallic acid and glucose which is required by the bacteria for its growth. Tannase production & tannin degradation was dependent on growth and resting cells could hardly degrade tannin (results not shown).

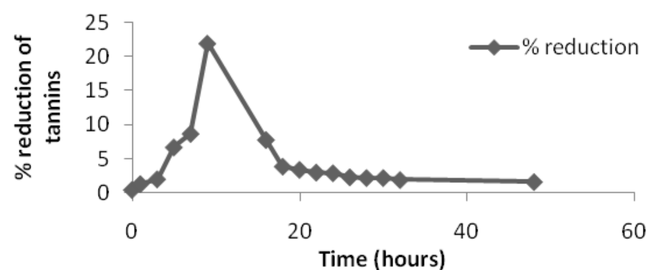

Figure 3. Kinetics of tannic acid degradation by *L. lactis*

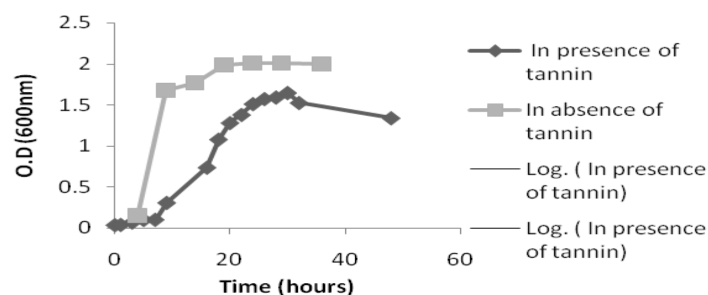

Figure 4. Growth kinetics of *L. lactis* in presence and absence of tannin

3.3 Tannase production

Tannase catalyzes the hydrolysis of ester linkages of the tannic acid producing gallic acid and thus the tannase activity could be measured by gallic acid estimation. A method proposed by (Deschamps *et al.,* 1983) was used for quantification of tannase activity where absorbance was read at 260 nm and amount of gallic acid produced in the reaction mixture was estimated from the standard curve of gallic acid. The enzyme production was inducible in nature and was found to be produced both intracellularly and extracellularly, while we tested for the activity of extracellular tannase and the results are shown in Figure 5.

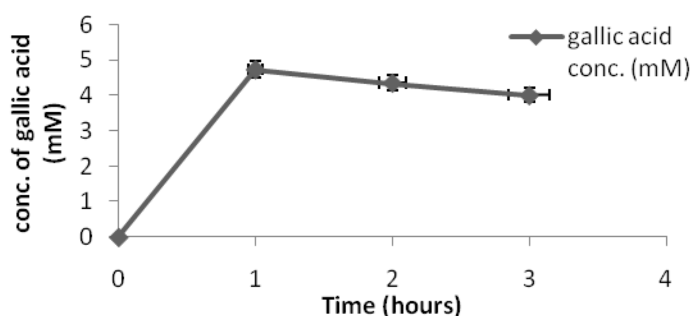

Figure 5. Quantification of tannase activity by gallic acid estimation

3.4 Effect of culture variables on L. lactis tannase

The effect of varying conditions on *L. lactis* tannase was estimated by the procedure of (Makkar *et al*., 1993). Tannase activity was measured by the reduction of tannin; hence, more the tannin reduction, higher is the tannase activity. The optimum temperature, pH and inoculums size for tannase activity was found to be 37˚C 6.8 and 1% inoculum respectively (Fig.6). Tannase activity was notably influenced by increase or decrease from optimal pH and agitation also affected tannase activity. Notable decrease in tannase activity occurred when culture was agitated at 120 rpm. Nitrogen source affected the tannase activity – absence of beef extract resulted in maximal tannin reduction (37%) followed by removal of peptone which showed a reduction of (16.7%) after 2 hours of incubation (Table 1). The above results were in concurrence to those suggested by other workers, barring that of pH, where our results indicated maximal activity at pH 6.8. The tannase activity remained unaffected at higher temperature (data not shown) indicating it to be thermostable.

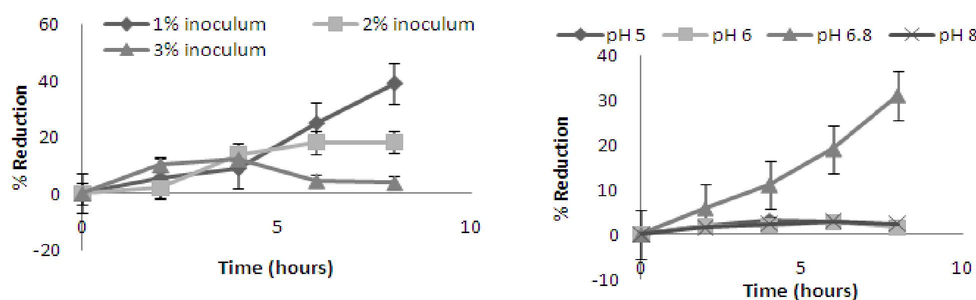

Figure 6.(A). Effect of inoculums size and **(B)** pH on tannase activity

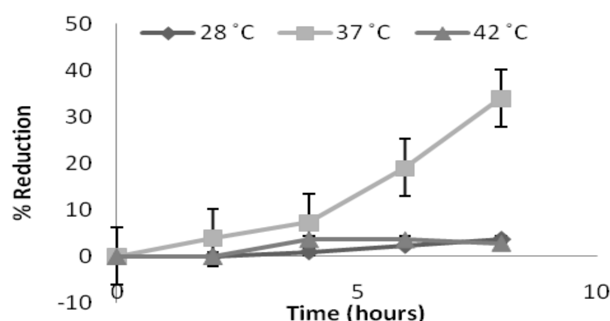

Figure 6.(C) Effect of different temperature on tannase activity

Table1. Effect of agitation and varying nitrogen source on tannase activity

	% reduction at 0 hour incubation	% reduction after 2 hour incubation	% reduction after 4 hour incubation	% reduction after 6 hour incubation
MRS without Beef extract	0	37	4	2
MRS without Peptone	0	16.7	4.08	2.16
MRS without Yeast extract	0	2.6	6.5	4
Agitation 120 rpm	0	2	4	7
No agitation	0	6.7	26.4	35.6

3.5 Tannin estimation in composite sourdough

Complete reduction of tannin (100%) was observed following 4 hours of fermentation, moreover a 72% reduction in saponins was attained. Importantly, tannase activity was not affected by the sourdough matrix which may have resulted in completion of tannin degradation thus emphasizing the applicability of *L. lactis* to be a potent strain for reducing antinutritional components.

Overall, the results of this study provided a firsthand evidence of thermostable tannase by an indigenous isolate of *L. lactis* subsp *lactis*. To the best of our knowledge, tannase production by *L. lactis* has not been reported thus far. Our previous work has demonstrated the probiotic and other technological benefits of *L. lactis* especially in composite sourdough which may provide enhanced nutritional benefits economically (Bhanwar *et al.*, 2012). The results of this study demonstrated a complete reduction of at least two major antinutrients in the sourdough when *L. lactis* is used as a starter culture. Currently, we are characterizing the biochemical aspects of *L. lactis* tannase and its implication in developing low cost, nutritive traditional foods are being explored further.

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