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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.

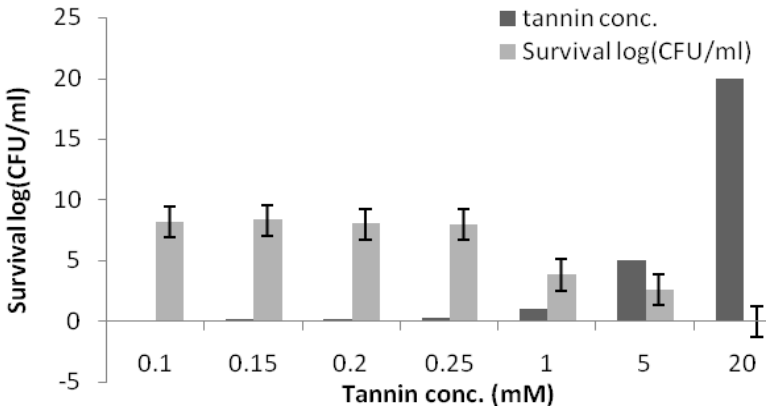

Figure 1. Tolerance to tannin

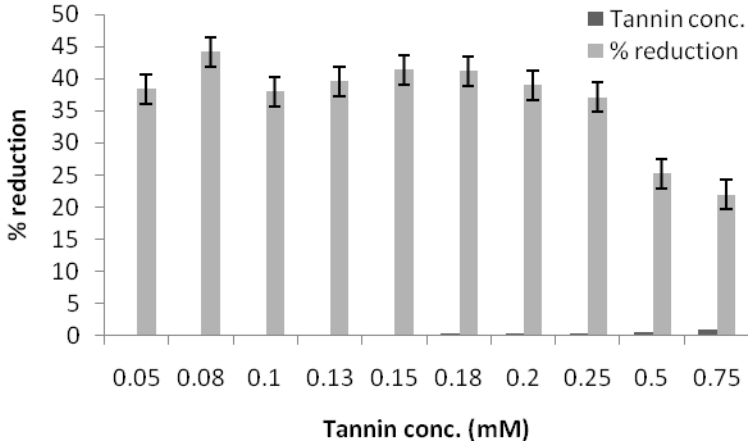

Figure 2. Reduction of tannin by whole cells

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).

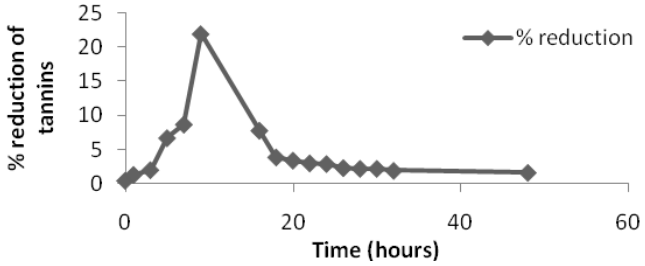

Figure 3. Kinetics of tannic acid degradation by *L. lactis*

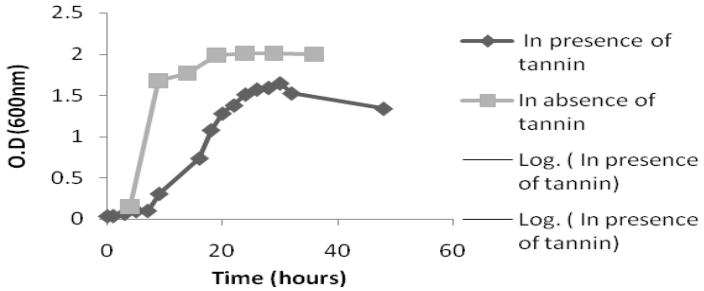

Figure 4. Growth kinetics of *L. lactis* in presence and absence of tannin

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.

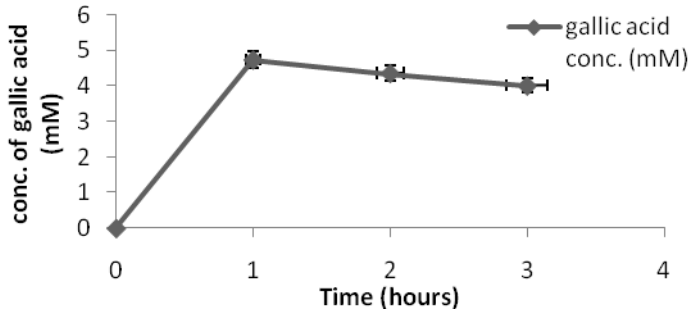

Figure 5. Quantification of tannase activity by gallic acid estimation

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.

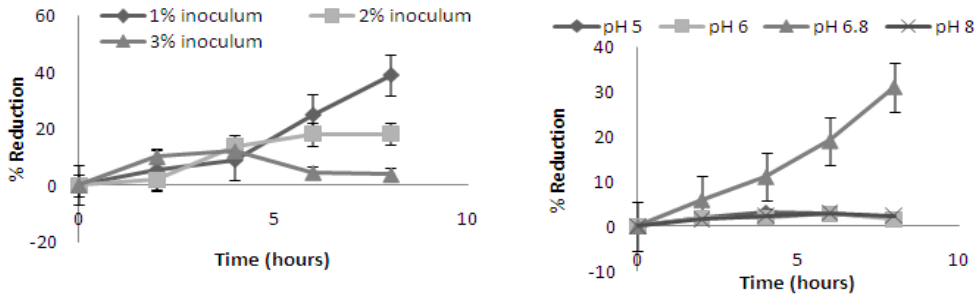

Figure 6.(A). Effect of inoculums size and **(B)** pH on tannase activity

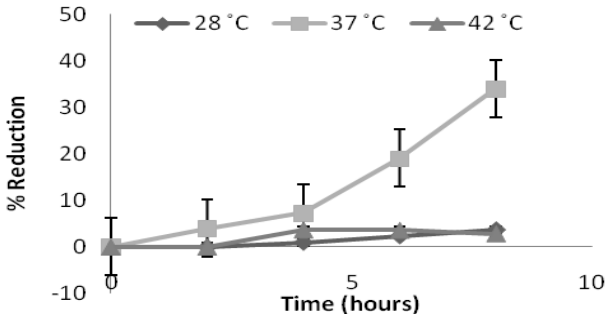

Figure 6.(C) Effect of different temperature on tannase activity

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.

5. Acknowledgement

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References

[1] E C Bate-Smith and T Swain (1962), *Flavonoid compounds in Comparative Biochemistry*, Academic press, pp. 705-809, vol 3A.

[2] S Bhanwar, M Bamnia, M Ghosh and A Ganguli (2012), Use of *Lactococcus lactis* to enrich sourdough bread with -aminobutyric acid, *Intl. J. Food Sci Nutr*. pp. 1-5.

[3] C Cuadrado, G Hajos, C Burbano, G Ayet, M Muzquiz and E Gelencser (2010), Effect of natural fermentation on the Lectin of Lentils measured by immunological method, *Food Agric Immunol*. pp. 41-49.

[4] A M Deschamps, G Otuk and J M Lebeault (1983), Production of tannase and degradation of chestnut tannins by bacteria. *J. Ferment Technol*. 61, pp. 55-59.

[5] P K Lekha and B K Lonsane (1997), Production and application of tannin acyl hydrolase, *Advances in applied microbiology,* 44. pp. 216-260.

[6] H P S Makkar, M Blummel, N K Borowy and K Becker (1993), Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J. Sci. Food Agr.* 61, pp. 161-165.

[7] H P S Makkar (1989) Protein precipitation methods for quantitation of tannins: A review, *J. Agric. Food Chem.* pp. 1197-1202.

[8] K C Mondal, D Banerjee, M Jana and B R Pati (2001), Colorimetric assay method for determination of the Tannin Acyl Hydrolase (EC 3.1.1.20) activity, *J. Anal Biochem.* pp. 168-171.

[9] K Poutanen, L Flander, K Katina (2009), Sourdough and cereal fermentation in a nutritional perspective, *J. Food Microbiol*. pp. 693-699.

[10] K N Suseelan, C R Bhatia and R Mitra (1997), Characterization of two major lectins from mungbean (*Vigna radiata*) seeds, *Plant food hum nutr*. 50. pp. 211-222.

[11] Y Uematsu, K Hirata and k Saito (2000), Spectrophotometric determination of saponin inYucca extract used as food additive. *J. AOAC Int.* 83. pp. 6.