



# Proteinase survey pursuit of *Lactobacillus plantarum* NCIM 2083

M. Y. Marathe and J. S. Ghosh\*

Department of Microbiology, Shivaji University, Vidyanagar, Kolhapur-416004, Maharashtra, India.

Abstract

Lactobacilli are important organisms used in several lactic acid fermentations. Lactobacilli cultured in milk perform activities like breaking down of lactose to simple sugars, proteolysis, lipolysis and lactic acid production. *Lactobacillus* depends on proteolytic system for degradation of protein – casein in milk from which they get their complex nutrient requirements for growth. This complex proteolytic system consists of proteinases, peptidases and amino and peptide carriers. Yogurt fermented milk having increased nutritive value, is more palatable, easily digestible and assimilable than milk. It has been observed that many people are sensitive to milk and cannot digest it easily. In developing nations this is an important cause for concern as such sensitive people suffers from protein-energy deficiency, more so if they are from the economically weaker section of the society. In this study an attempt has been made to understand the proteinase system from *Lactobacillus plantarum* NCIM 2083. The enzyme was found to be cell wall bound. It was active optimally at 30°C at pH 7.0. The peptides obtained after proteolysis were studied using SDS- PAGE, HPLC techniques and Liquid chromatography mass spectrometry (LCMS).

**Keywords:** Proteinase, *lactobacillus*, yogurt, casein, peptides.

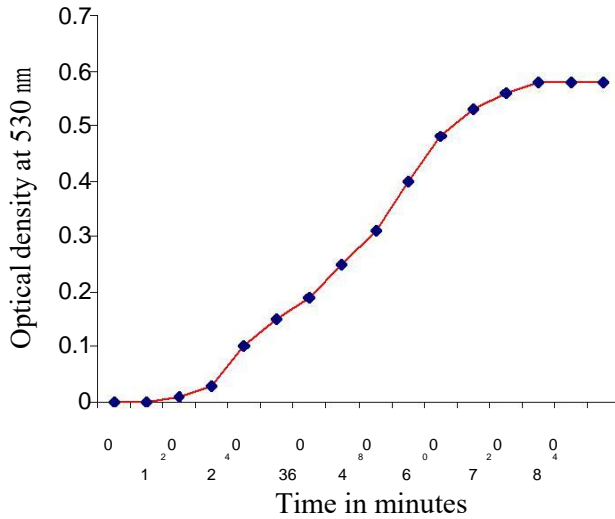
## INTRODUCTION

*Lactobacillus* is a genus of gram-positive facultative anaerobic or microaerophilic bacteria belonging to the family Bacillaceae (Sneath, 1994). They convert lactose and other sugars to lactic acid. These are fastidious organisms as they require complex nutrients like amino acids, peptides, vitamins for growth. Since the concentrations of free amino acids and peptides are very low in milk, they depend for growth in milk on a complex proteolytic system, mostly cell wall bound and consisting of proteinases, peptidases including proline specific peptidases (Geis et al., 1985) and carriers of amino acids and peptides (Tsakalidou et al., 1999) for degradation of milk protein into peptides, a large part of the resulting peptides is taken up via the oligopeptide transport system (Kunji et al., 1996). Further degradation to amino acids is mediated by a set of peptidases (Christophe, 1997; Marrug et al., 1995). Similar proteinases have been reported from *Lactococcus lactis* ssp *cremoris*, *Lactobacillus delbrueckii* ssp *bulgaricus* and *Lactobacillus casei* (Soda, 1986; Shin, 2004), *L. bulgaricus* (Courtin et al., 2002), *Lactobacillus rhamnosus* (Pastar et al., 2003), *lactobacillus paracasei* (Bintsis et al., 2003), *Lactobacillus helveticus*, *L. delbrueckii* (Oberge et al., 2002; Germond et al.,

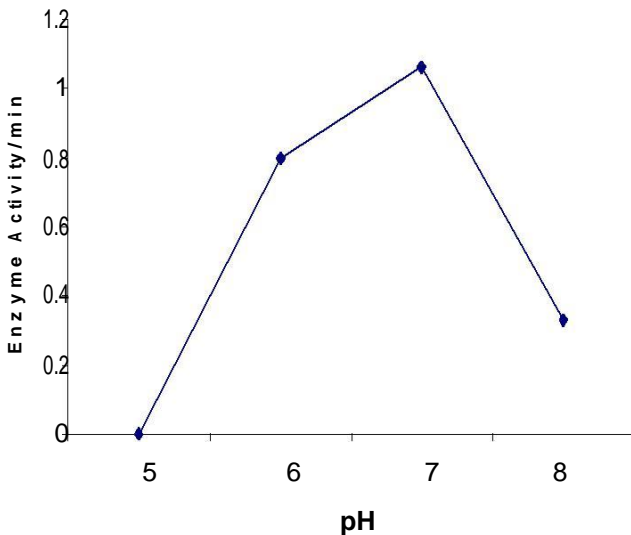
2003), *Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum* and *Lactobacillus plantarum* (Mugula et al., 2003).

Proteolytic system of Lactic Acid Bacteria (LAB) has been mainly studied with reference to cheese technology, as it plays an important role in the texture and flavor development of cheese (Courtin et al., 2002; Wilkinson, 1995).

*Lactobacillus* is used in the dairy industry with other lactic acid bacteria (LAB) to produce fermented milk, sour milk and yogurt (Elvira et al., 2001). The demand for processed dairy foods has increased considerably with growing urbanization, especially for different cheese varieties and low-lactose milk. Proteinase also helps to reduce the allergic properties of milk and milk products for infants which can lead to a severe nutritional problem of protein-energy deficiency (Yuan, 2009). It has been estimated that fermented milk products including yogurt has increased nutritive value as compared to the market milk (De, 2005). Casein is the principal protein fraction of cattle milk along with lactalbumin and lacto globulins. These account for more than 80% of the protein content and are present in concentrations of 2.5 - 3.2% (De, 2005). It is the most common and economically affordable source if pro-



**Figure 1.** Growth curve of isolated *Lactobacillus* spp.



**Figure 2.** Activity of proteinase enzyme at different pH values.

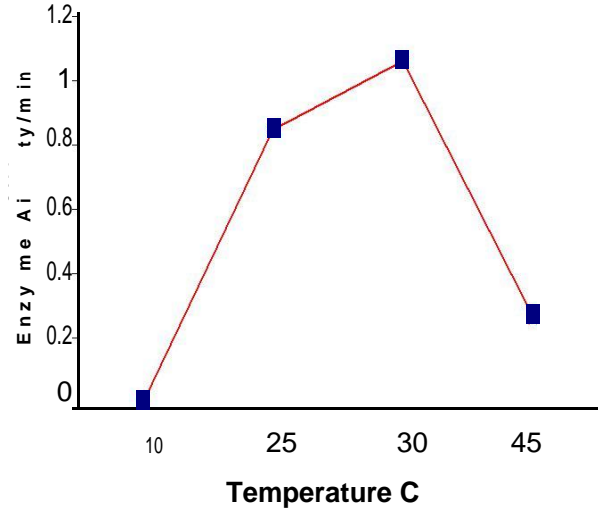
of protein for humans all over the world.

#### **MATERIALS AND METHODS** Micro-organisms and culture conditions

Growth pattern of *L. plantarum* NCIM 2083 was studied in MRS (Atlas, 2004) medium at room temperature and the results are as shown in Figure 1.

#### **Preparation of cell wall extract**

The organism was grown in modified mineral based medium having the following composition per litre: casein - 10 g, NaNO<sub>3</sub> - 3 g, Cells were collected and washed two times with 50 mM phosphate buffer (pH 7.0) containing 20 mM CaCl<sub>2</sub>. Washed cells K<sub>2</sub>HPO<sub>4</sub> - 1



**Figure 3.** Activity of proteinase enzyme at different temperatures.

g, MgSO<sub>4</sub> - 0.5 g, KCl - 0.5 g, FeSO<sub>4</sub> - 0.01 g and the final pH was adjusted to 6.0. were resuspended in the same buffer but without CaCl<sub>2</sub> and incubated for 2 - 3 h at 30°C. The supernatant obtained after centrifugation at 8000 rpm at 4°C for 20 min was designated as cell wall extract (Tsakalidou et al., 1999).

#### **Assay of proteinase activity**

Cell wall extract (100 µl) was incubated with 100 µl of casein solution (4 mg/ml) for 24 h at 30°C in 200 µl of phosphate buffer (50 mM) of pH 7. The reaction was stopped by addition of 400 µl of 12% trichloroacetic acid (TCA) and further incubated for 10 min at 25°C. This was then centrifuged at 8000 rpm for 10 min to remove all insoluble matter and it was assumed that the clear solution would contain mostly soluble peptides and some amino acids only. This supernatant was analyzed by Lowry method (Lowry et al., 1951). One unit of proteinase activity was defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 660 nm under above mentioned conditions.

#### **Effect of pH on proteinase activity**

The above assay was carried out with buffers of different pH values like 5, 6, 7 and 8. The results are as shown in Figure 2.

#### **Effect of temperature on proteinase activity**

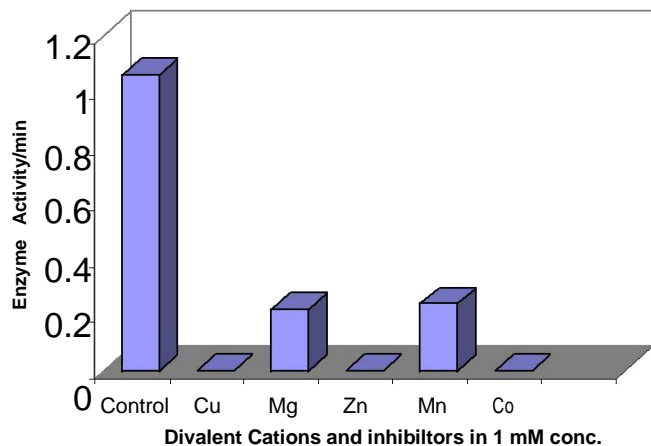
The proteinase activity was similarly assayed at different temperatures of incubation ranging from 10 - 40°C. The results are as shown In Figure 3.

#### **Effect of divalent cations**

Divalent cations like Cu<sup>++</sup>, Co<sup>++</sup>, Hg<sup>++</sup>, Zn<sup>++</sup>, Mn<sup>++</sup> and Mg<sup>++</sup> at concentration of 1 mM were added separately to the assay mixture and the activity was recorded. The results are as shown in Figure 4.

#### **Polyacrylamide gel electrophoresis (PAGE) of the hydrolysate**

Cell wall (1 ml) extract was incubated with 1 ml of casein solution



**Figure 4.** Shows effect of divalent cations on Enzyme activity.

(0.5 mg/ml) for 4 h in 2 ml of 50 mM phosphate buffer (pH 7) at 30°C. The reaction was stopped by addition of 8 ml of 12% TCA. Centrifugation was carried out at 8000 rpm for 10 min. The supernatant obtained was concentrated by dialysis. This concentrate was mixed with sample buffer in 1:1 ratio and heated for about 5 min in boiling water bath and analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970). The results are as shown in Figure 5.

#### High performance liquid chromatography (HPLC) analysis

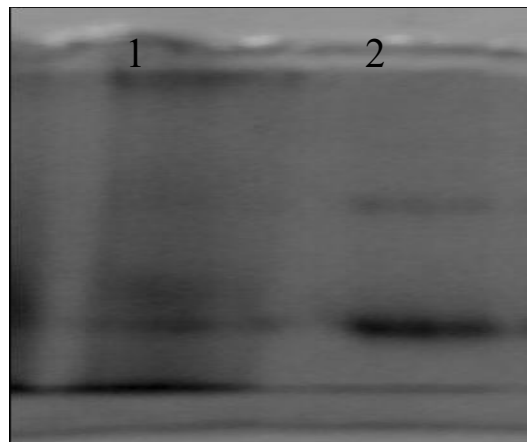
Cell wall extract (0.5 ml) was incubated with 0.5 ml of casein (0.5 mg/ml) in phosphate buffer (50 mM) of pH 7.0 at 30°C for 4 h. The reaction was stopped by addition of 12% TCA (1 ml). After 10 min at 25°C, the sample was centrifuged (8000 rpm for 10 min). The supernatant was concentrated and redissolved in methanol and used for further analysis. The peptides were separated on Nucleosil C<sub>18</sub> column and detected by absorbance at 220 nm (Tsakalidou et al., 1999). The results are as shown in Figure 6.

#### Peptide identification by Liquid Chromatography Mass Spectrometry (LCMS)

Methanolic solutions of casein protein hydrolysates obtained as mentioned above, after 4 and 24 h of incubation, were subjected to Liquid chromatography mass spectrometry. The results are as shown in Tables 1 and 2.

## RESULTS AND DISCUSSION

It can be seen from Figure 1 that the exponential phase of growth is between 3 - 12 h. However, the proteinase activity was maximum at 10 h of incubation. The pH Further confirmation was done by LCMS analysis where in it can be seen that the protein were being degraded by 4 h of incubation but is more prominent after 24 h of incubation as seen by near multiples of MW 280.89. Optima were at 7.0 and the optimum temperature for maximum activity was at 30°C. The PAGE bands showed lot of undegraded casein after 4 h of incubation but after removing the undegraded casein the degraded products



**Figure 5.** Polyacrylamide Gel Electrophoresis: Action of cell wall crude proteinase on casein (1) after 4 h - shows hydrolysate where casein was not separated out from free peptides. (2) Action of cell wall crude proteinase on casein after 4 h, casein was separated from peptides. Reaction mixtures were incubated at 35°C and pH 7.0.

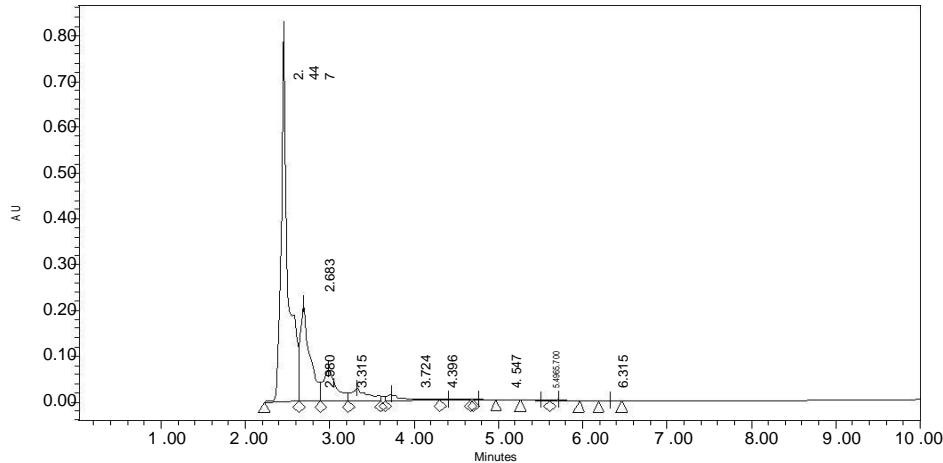
become more prominent. This is also confirmed on HPLC where undegraded casein was removed before the analysis. It may be observed that depending on the breakdown products there is a steady gradient in the retention time.

## Conclusion

In our country a lot of yoghurt is consumed without any added flavours of any type, but it is prepared at home and rarely purchased from any dairy processing units. The starter culture used in this fermentation is a small amount of previously yogurt. Over a period of time the fermentation has been observed to be mostly by heterofermentative coliforms and not by *Lactobacillus* spp. Therefore, the contents of fermentation of milk to yogurt are not known and it varies from family to family. However, if a good yogurt has to be prepared with all the nutritional values then it would be better if cultures of *Lactobacillus* especially *L. plantarum* should be made available for domestic use. We have also found out that the method of curdling is well known (preheating of milk and then adding the starter at around 40°C and holding it for 12 - 18 h). Therefore, fermentation by lactobacilli should be easily accepted by every household. Milk proteins contain all essential amino-acids in fairly large quantities. If people in developing country like ours can make partially digested milk protein through yogurt, then it will reduce the protein deficiencies and related metabolic disorders.

## ACKNOWLEDGMENT

This work is in fact a part of the master's degree in micro-



**Figure 6.** High performance liquid chromatography studies of peptides obtained after 4 h of enzyme action on casein (0.5 mg/mL).

**Table 1.** Peptide Mass Identification by LCMS after 4 h of incubation of enzyme with casein (0.5mg/mL).

Sr. No.	Molecular Weight (M+1)
1	1570.94
2	736.7
3	1187.96
4	590.92
5	1593.43
6	1163.3
7	1160.38

**Table 2.** Peptide Mass Identification by LCMS after 24 h of incubation of enzyme with casein (0.5 mg/mL).

Sr. No.	Molecular Weight(M+1)
1	1929.97
2	282.83
3	736.48
4	280.89
5	1442.05

biology of our university. We are very grateful to Professor S.P. Govindwar – Professor and Head, Department of Biochemistry for providing all the laboratory facilities towards successful completion of this work.

## REFERENCES

Atlas RM (2004). Handbook of microbiological media. Third edition CRC press, New York. p. 1209.  
 Bintsis T, Vafofoulou- Mastrojiannaki A, Litopouloutzanetaki E, Robinson RK. (2003). Protease, Peptidase and Esterase Activities

by Lactobacilli and Yeast isolates from feta cheese brine. J. Appl. Microbiol. 95(1): 68–77.

Christophe G, Brigitte B, Jacques P, Daniea A (1997). Comparison of cell surface proteinase activities within the *Lactobacillus* genus. J. Dairy Res. 64: 561-571.

Courtin P, Nardi M, Wegmann U, Joutsjoki V, Ogier JC, Gripon JC, Palva A, Henrich B and Monnet V (2002). Accelerating cheese proteolysis by enriching *Lactobacillus lactis* proteolytic system with *Lactobacillus* peptidases. Int. Dairy J. 12: 447-454.

De S (2005). Market milk, Outlines of Dairy Technology. Oxford Press. pp. 12-13.

De S (2005). Indian Dairy Products, Outlines of Dairy Technology. Oxford Press. pp. 404-405.

El Soda M, Desmazeaud MJ, Bars Zevaco C. (1986). Cell-wall associated proteinases in *Lactobacillus casei* and *Lactobacillus plantarum*. J. Food Prot. 49: 361-365.

Elvira MH, Graciela S, De Giori Raul RR (2001). Isolation and Characterization of a Slowly Milk-Coagulating Variant of *Lactobacillus helveticus* Deficient in Purine Biosynthesis. Applied & Environ Microbiol. 67(4): 1846–1850.

Geis A, Bockelmann W, Teuber M (1985). Simultaneous extraction and purification of cell wall associated peptidase and -casein specific protease from *Streptococcus cremoris* AC1. Appl. Microbiol. Biotechnol. 23: 79-84.

Germond JE, Delley M, Gilbert C, Atlan D. (2003). Determination Of The domain Of the *Lactobacillus delbrueckii subsp. bulgaricus* Cell Surface Proteinase Prtb involved in attachment to the cell wall after Heterologous expression of the Prtb gene in *Lactococcus lactis*. Appl. Environ. Microbiol. 69(6): 3377-3384.

Germond M, Fira D Banina A, Topisirovic L (1991). Characterization of the cell wall bound proteinase of *Lactobacillus casei* HN14. Appl. Environ. Microbiol. 57: 1753-1757.

Kunji ER, Meierau I, Hagting A, Poolman B, Konings WN (1996). The proteolytic system of Lactic acid bacteria. Antonie van Leeuwenhoek. 70: 187-221.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bactriophageT4. Nature (Lond.). 227: 680-685.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mugula JK, Sorhaug T, Stepaniak L. (2003) . Proteolytic activities in Togwa, a tanzanian fermented food. Int. J. Food Microbiol. 84(1): 1-12.

Oberg CJ, Broadbent JR, Strickland M, Mcmohan DJ. (2002). Diversity in the Specificity of the Extracellular Proteinases In *Lactobacillus helveticus* and *Lactobacillus delbrueckii subsp. bulgaricus*. Lett.

- Appl. Microbiol. 34(6): 455-460.
- Pastar I, Tonic I, Golic N, Kojic M, Van Kranenburg R, Kleerebezem M, Topisirovic L and Jovanovic G (2003). Identification and genetic characterization of Novel Proteinase, Prtr, from the human isolate *Lactobacillus rhamnosus* bgt10. Appl. Environ. Microbiol. 69(10): 5802-5811
- Shin JY, Jeon WM, Kim GB, Lee BH. (2004). Purification and Characterization of Intracellular Proteinase from *Lactobacillus casei* ssp. *casei* LLG. J. Dairy Sci. 87: 4097-4103
- Sneath PHA (1994). Endospore- forming gram-positive rods and cocci. In Bergey's manual of Systematic Bacteriol 2: 1104- 1139.
- Tsakalidou E, Anastasiou R, Vandenberghe I, van Beeumen J, Kalantzopoulos G. 1999. Cell wall bound proteinase of *Lactobacillus delbrueckii* subsp. *Lactis*. ACA- DC178: Characterization & specificity for beta-casein. Applied & Environ Microbiol. 65: 2035-2040.
- Wilkinson MG (1995). Cheese – Chemistry. Physics Microbiology – General Aspects (ed. Fox PF, Chapman and Hall. London. 1: 523-555.
- Yuan Q (2009). Insight into milk protein allergy: Microenvironment matters. Gastroenterology 124 (1): 259-261.