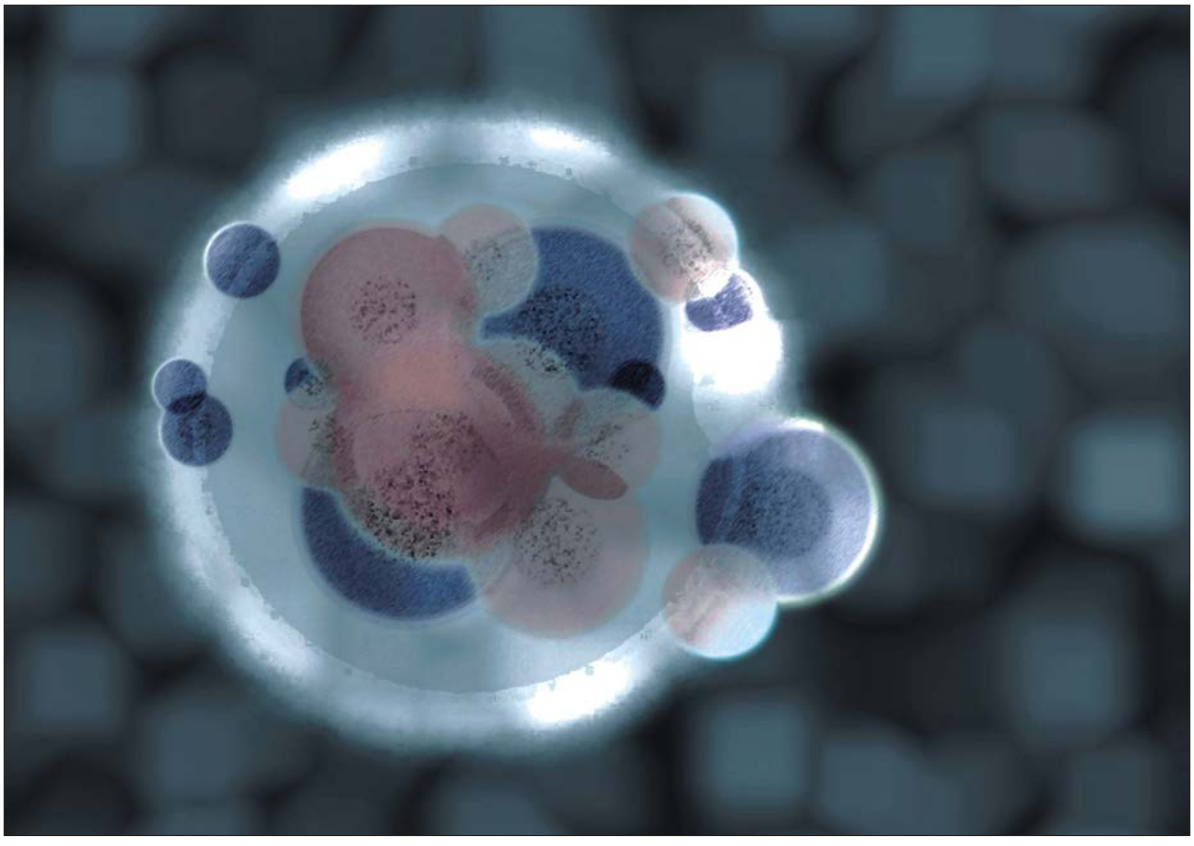

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Research Article
Characterization of Protease Activity of *Lactococcus lactis* Species Isolated from Raw Camel's Milk

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Abstract

Background and Objective: The effect of physical and nutritional factors on the protease activity of lactic acid bacteria was evaluated by overlay assay under different temperatures, pH and modified growth medium. The aim was to find better conditions for the proteolysis of new indigenous lactic acid bacteria strains isolated from raw camel's milk and this in order to develop new flavors for dairy products. **Materials and Methods:** Strains including *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus garviae* were isolated from raw camel's milk and screened for the optimization. **Results:** The results showed that *Lactococcus* strains had a good potential of proteolysis and that maximal protease activity occurrence was after an incubation period of 24 h at a temperature of 37°C and a pH of 7.2. Among the different carbon sources, lactose and glucose seemed to be the best for all *Lactococcus* strains. Likewise, casein and yeast extract revealed as the optimal nitrogen sources for best protease enzyme production for all the tested isolates. Furthermore, the association of glucose and yeast extract was good for protease activity but the one of lactose and casein was the best. **Conclusion:** Best protease activity of *Lactococcus* species isolated from raw camel's milk was at 37°C after 24 h of incubation at pH 7.2 and in medium containing lactose (5 g L^{-1}) with casein (5 g L^{-1}).

Key words: Lactic acid bacteria, proteolysis, *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus garviae*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lactococci are Gram-positive bacteria used in the production of a great number of fermented dairy products due to their efficient proteolytic and transport systems to degrade milk casein. This catabolism contributes to the development of flavors in fermented dairy products[1-3]. Many studies showed that proteinase production is mainly dependent on the medium composition. Therefore, high rates of proteinase production were found in milk, better than in laboratory culture media, rich in peptides and amino acids[4-6]. Moreover, studies have showed that nutritional factors including carbon and nitrogen sources can influence the protease enzymes production. Besides these nutritional factors, physical factors such as temperature and pH can also effectively influence the protease enzymes production[7]. The main purpose of the present study is the optimization of protease activity of different *Lactococcus* species (sp.) (*Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus garviae*) isolated from camel's raw milk. This kind of milk was chosen to essay introducing new strains in industry of dairy products because his exploration was poor in the past with possibility of developing new flavors to those products.

MATERIALS AND METHODS

Culture conditions: Strains of Lactic Acid Bacteria (LAB), isolated from raw camel's milk were examined for protease enzymes production in skim milk agar plates. High protease producing strains have been selected. The M17 medium has been used for the isolation and the conservation of the isolates. Then, Api 50CH has been used for their identification.

Dosing of protease activity: Proteolytic activity has been assessed using casein as substrate. For this, overnight grown cultures of the studied strains have been centrifuged at 4600 rmp for 15 min at 4°C. Then, the protease activity has been evaluated. Only the culture supernatants were assayed for this. About 0.5 mL of enzyme solution (Culture supernatant) were added to 3 mL of substrate solution (Table 1) and the mixture has been incubated at 30°C for 20 min than 1 mL of ninhydrin-$CdCl_2$ mixture was added. The reaction has been stopped by adding 3 mL of TCA mixture followed by heating of 5 min at 84°C and cooling in ice bath for 10 min. A filtration through Whatman filter paper No. 1 has been done. Then, the absorbance of the filtrate has been measured at 507 nm.

Table 1: Composition of used solutions for testing protease activity

Solution	Composition
Substrat solution	0.6% casein in 0.1 M tris-HCl buffer, pH 8
Ninhydrin-$CdCl_2$ mixture	1 g of $CdCl_2$ in 1 mL of distilled water+0.8 g ninhydrin in 80 mL ethanol+10 mL acetic acid
TCA mixture	0.11 M trichloroacetic acid+0.22 M sodium acetate+0.33 M acetic acid

Protease activity at different temperatures: The M17 broth has been used for the incubation of LAB strains at different temperatures (28, 30 and 37°C) for 24 h. Thereafter, the supernatants were analyzed for protease activity to evaluate the effect of temperature on the protease enzymes production.

Protease activity in different pH: The M17 broth previously adjusted to different pH values (5.5, 7.2 and 8) using 1N HCl and 1N NaOH solutions has been used for cultivation of the studied strains. After an incubation of 24 h at 30°C, the supernatants were analyzed for protease activity to assess the effect of pH on this activity.

Protease activity in modified M17 medium: To evaluate the effect of different carbon, nitrogen and protein sources on protease activity in culture media, glucose and sucrose (Carbon sources), yeast extract, glutamic acid, peptone and potassium nitrate (Nitrogen sources) and casein and gelatin (Protein sources) were added to M17 broth. Following an incubation of 24 h at 30°C and the supernatants were analyzed for protease activity.

Determination of the optimal casein content: The optimal casein content has been determined by introducing different concentrations of casein to the culture media under optimized conditions. The casein concentrations used in this test were between 0 and 10 g L^{-1}. The inoculated media with different casein concentrations have been incubated at 37°C for 24 h and the total proteolytic activity has been then measured to determine the optimal casein content.

RESULTS

Selection of strains: High protease producing strains have been selected in this study based on their ability to produce clear zone on skim milk agar (Fig. 1). Therefore, out of 90 isolates, belonging to three strains namely *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus garviae* have been selected. The results indicated that the protease activity was most likely extracellular which is clearly shown by the clear zones given the by the supernatants in skim milk agar.

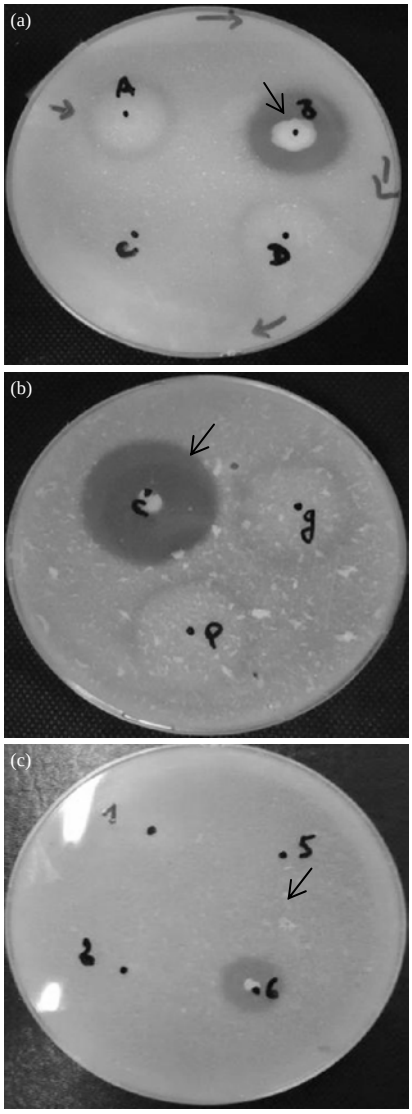

Fig. 1(a-c): Clear zones of proteolytic strains on skim milk agar by (a) *Lactococcus lactis*, (b) *Lactococcus cremoris* and (c) Lactococcus garviae

Protease activity under different temperatures: The studied strains had a good protease activity (Good values) and similar behaviors in response to the variation of the temperature (Table 2). Furthermore, considerable variation of the protease activity has been noted for all the strains in function of temperature changes (4, 28, 30 and 37°C) with maximum rate at 37°C followed by the one at 28°C. Then, the activity decreased at 30°C and became negligible at 4°C (Table 1).

Protease activity under different pH: The pH had a significant effect on the protease activity. Moreover, the studied strains showed similar reactions to the pH variation. Therefore, an increase in the pH from 5.5-7.2 accompanied by an increase in protease activity however, another increase in pH from 7.2-8 conduced to a decrease in protease activity (Table 2).

Table 2: Protease activity under modified temperatures, pH and M17

Protease activity (mg mL^{-1}) strains	*Lactococcus lactis*	Lactococcus cremoris	*Lactococcus garviae*
Temperature (°C)			
4	1.24	2.06	1.80
28	37.69	46.83	37.91
30	35.06	44.65	35.75
37	38.89	47.06	42.65
pH			
5.5	20.76	19.02	18.49
7.2	45.05	38.06	36.95
8	24.76	23.41	23.12
Modified M17			
Glucose	35.44	43.00	34.51
Sucrose	33.76	42.68	33.75
Casein	41.30	47.44	40.60
Gelatin	20.60	23.32	19.48
Glutamic acid	11.24	11.22	10.53
KNO_3	25.84	28.30	22.12
Peptone	34.75	37. 30	33.45
Yeast extract	38.65	39.80	36.00

These results demonstrated that 7.2 is the optimum pH for the protease activity and that higher or lower pH values may lead to partial loss in protease activity by *L. lactis*, *L. cremoris* and *L. garviae*. Moreover, pH 5.5 (Acidic conditions) affected more severely the protease activity than pH 8 (Basic conditions).

Protease activity in modified M17 medium: Best protease activity has been obtained, when the culture medium was supplemented with glucose for all strains (Table 2). Those results are closer to the values obtained with the M17 medium which contains the lactose (Glucose+galactose). This activity has slightly decreased when the sucrose has been used as carbon source.

Likewise, when the medium was supplemented with casein, protease activity was on its peak (Table 2) and it was minimal with gelatin as protein source. Concerning the nitrogen sources, the protease activity was maximal with yeast extract (Table 2) moderate to good rates were obtained with peptone. Nevertheless, the activity has decreased when KNO_3 and glutamic acid were used as nitrogen sources.

Determination of optimal casein content: The protease activity of *L. lactis*, *L. cremoris* and *L. garviae* was on its peak with casein concentration of (5 g L^{-1}) (Fig. 2). A decrease in protease activity was observed for all strains when the casein content was over or less than this value.

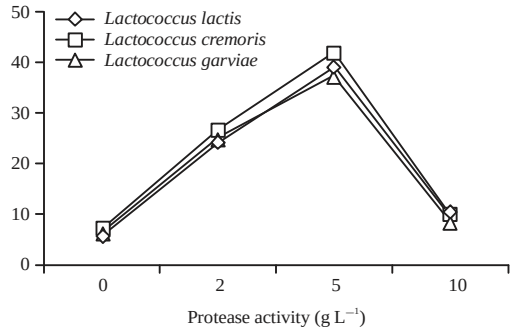

Fig. 2: Protease activity under different casein content of the medium by *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus garviae*

DISCUSSION

Protease activity was most likely extracellular, it was proved by the clear zones given by supernatants on skim milk agar plates. That is in accord with results of El-Ghaish *et al.*[8] who indicated that proteolytic cocci isolates of lactic acid bacteria gave clear zones on skim milk agar. Protease activity was also extracellular and that is in accordance with the results of Nicodeme *et al.*[9] who studied the extracellular protease activity of different supernatants of *Pseudomonas* strains.

Lactococcus species showed good values of protease activity Requena *et al.*[10], Quigley *et al.*[11] and Terzic-Vidojevic *et al.*[12] also reported that the cell-wall membrane of *L. lactis* sp., *lactis* has the highest proteolytic activity compared with *lactobacilli*. This is also in accordance with results of Gonzalez *et al.*[13] who compared between strains of *Lactococcus lactis* sp., *Lactis*, *Leuconostoc*, *Lactobacillus* and *Entrococcus faecalis* and reported that Cell Freee Extract (CFE) of a strain of *Lactococcus lactis* sp., *Lactis* GE 2363 has the highest level of proteolytic and caseinolytic activity.

Temperature 37°C was best for protease activity and further decrease or increase of temperature, led to significant decrease in the proteolytic activities. Results of Gobbettia *et al.*[7] also showed that the protease activity of *L. lactis* sp., *lactis* T12 was on top between 35 and 40°C. Corresponding of that El-Ghaish *et al.*[8] found that the optimum temperature of protease activity was at the range of 15 and 45°C. However, Terzic-Vidojevic *et al.*[12] and De Giori *et al.*[14] indicated that the highest proteolytic activity of *E. faecalis* HH22 and *E. faecium* DO623 was observed at 42°C and that the one of *Lactobacillus plantarum* was at 47°C and that further decrease or increase of the incubation temperature led to significant decrease in the proteolytic activities.

The pH 7.2 was best for protease activity of strains and that is in agreement with results of Gobbettia *et al.*[7] who reported that pH 7 was the optimum pH for proteolytic activity of *L. Lactis* sp., *lactis* T12, it was then (42 U mg^{-1}) compared to (12 U mg^{-1}) when the pH was between 5.0 and 5.5. The activity has also reduced when the NaCl concentration increased. Results of Marugg *et al.*[5] also showed that pH 7 was the optimum pH of *L. cremoris* for production of aminopeptidases[12] also showed that production of endopeptidases of *Lactobacillus plantarum* was at his optimum at pH 7 and that a further decrease or increase of pH led to a decrease of protease's production, El-Ghaish *et al.*[8] also reported that maximal protein degradation was observed in the range pH of 6.5-7.2 for *E. faecalis* HH22 and at pH 6.5 for *E. faecium* DO623. Further, the increase in pH led to a decrease in proteolysis. However, the extent of hydrolysis at basic pH 7.2-8.2 was higher than in acidic pH 5.7-6 for both strains. This study concluded that the optimal pH of the proteolytic enzymes of these bacterial strains is 6.5 and that basic conditions are more favorable for proteolysis than acidic conditions and that is in accord with these results.

These results demonstrated that glucose was the best carbon source for protease activity and that is in accord with results of Brzozowski and Lewandowska[15] who demonstrated that glucose was the best carbon source for production of aminopeptidases of *Lactobacillus acidophilus*, Furthermore, Herbert *et al.*[16] and Wehrle *et al.*[17] mentioned that glucose was the best carbon source for protease activity of *Bacillus* sp. and that it is followed by sucrose.

Best results of proteolysis were obtained when casein was added to the medium. Smid *et al.*[1] also reported that the cell envelope of *Lactococcus lactis* contains a caseinolytic proteinase which is the first enzyme in the pathway of casein catabolism and that the growth of *L. lactis* was at its optimum when the media contains casein. Regarding the nitrogen sources, yeast extract and peptone which are present in culture medium, gave good values of proteolytic activity while, it decreased with glutamic acid and KNO_3. Marugg *et al.*[5], Marugg *et al.*[6], Hebert *et al.*[16] and Boominathan *et al.*[18] also found that protease activity rates of *L. lactis* sp. and *Lactobacillus* sp. was observed in growth medium and that yeast extract was the appropriate nitrogen source for protease activity it was followed by peptone. Studies of Marugg *et al.*[5], Marugg *et al.*[6] and Hebert *et al.*[16] also showed that addition of specific peptides to the medium of growth of *Lactococcus lactis* and *Lactobacillus* sp., led to a decrease in protease activity.

The optimal concentration of casein for best protease activity was (5 g L^{-1}) as described by Nour *et al.*[19] who studied protease activity of *Lactobacillus bulgaricus*. Marugg *et al.*[5] and Marugg *et al.*[6] also reported that high levels of *prtP* gene (Encoding for extracellular protease) expression was found for *Lactococcus lactis* SK11, cultured in media with low peptides concentrations and that the expression of *prtP* is repressed by high nitrogen concentrations.

CONCLUSION

This study showed clearly that *Lactococcus* species had a good potential of proteolysis and that the preferred conditions for maximal protease activity of *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus garviae* isolated from raw camel's milk are an incubation period of 24 h at pH of 7.2, temperature of 37°C with (5 g L^{-1}) of casein content as protein source (5 g L^{-1}) of lactose as carbon source (2.5 g L^{-1}) of yeast extract as nitrogen source. Yeast extract at (2.5 g L^{-1}) as nitrogen source and glucose at (5 g L^{-1}) as source of carbon also shows good levels of protease activity. So, those indigenous strains can be introduced after culture in those conditions in industry of dairy products in order to give best proteolysis and with possibility to develop new flavors.

SIGNIFICANCE STATEMENTS

Optimization of protease activity of *Lactococcus* species isolated from camel's raw milk to give new flavors to fermented milk products and improvement of industry of dairy products.

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