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Study on the impact of thermal treatment of milk on coagulation efficiency of pumpkin seed protease

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Abstract
Plant-based enzymatic coagulants are becoming more and more popular in the dairy and allied food industries because they are easily accessible, comply with government regulations, have a high economic value, are easy to use, and are in line with vegetarian diets and expanding health trends. Therefore, the purpose of this study was to check the possibility of crude extract of pumpkin seed as a source of milk coagulating enzyme as well as the impact of thermal treatment of milk on its coagulation efficiency. The milk coagulation efficiency of the extract was significantly different for thermally treated and non-treated milk. Based on this study, it was found that the protease derived from pumpkin seeds exhibits the potential to coagulate milk over a broad temperature range. The coagulation efficiency for thermally treated milk was lower than for non-treated milk.

Keywords: Plant, enzyme, milk coagulation activity, pasteurisation, temperature

Introduction
The enzyme industry plays a vital role in various sectors, ranging from food and dairy to biotechnology and pharmaceuticals. In the food industry, enzymes are utilized for processes such as fermentation, baking, and brewing, enhancing flavour, texture, and nutritional value. In the dairy industry, enzymes contribute to cheese production and lactose breakdown (Mazorra-Manzano *et al*., 2018) [19]. As a result, the market demand for enzymes remains consistently high, regardless of geographical location. One of the most widely used types of enzymes in industrial settings is protein-degrading peptidases also named as proteases. Protease enzymes hold paramount significance, constituting approximately 62% of the entire enzyme production industry (Freitas *et al*., 2016) [9].

Rennet, a well-known animal-derived protease enzyme exploited mainly for cheese production in the dairy industry (Hickey *et al*., 2017) [13]. The traditional source of the rennet is the true / fourth stomach of a young calf. Chymosin is the principal enzyme in calf rennet or standard rennet (Ray & Rosell., 2017) [20]. The traditional way of producing involves slaughtering of calves and extracting the enzyme from their stomach (Harboe *et al*., 2010) [11]. However, the yield of the product is too small and inadequate to meet the demand for the cheese industry. Owing to the limited availability, the cost of rennet increased. Moreover, People object to the use of animal products in cheese for religious reasons (Saxena & Sasmal., 2021) [22]. The investigation was directed by the above-mentioned concerns, to explore an alternative, cost-effective source of plant-based milk-clotting enzymes that can be a satisfactory substitute of animal peptidase for milk coagulation and suitable in the production of different varieties of cheese and similar fermented dairy based products.

Plant proteases play diverse roles throughout the plant life cycle, contributing to various physiological processes such as growth of plant, senescence, nitrogen homeostasis, fruit ripening and programmed cell death (Sharma & Gayen *et al*., 2021) [24]. These enzymes are present in almost all kinds of plant tissues such as roots, leaves, fruits, seeds and flowers (Sun *et al*., 2016) [25]. Peptidases are broadly categorised into different groups according to their catalytic mechanisms employed in hydrolysis. The primary catalytic types include aspartate, serine, cysteine, and metalloproteases, but except metalloproteases others have been reported in literature to have significant milk coagulation efficiency (Shah *et al*., 2014) [23].

Two aspartic residues are embedded at the catalytic site of aspartic proteases and are maximal activity at lower pH. Aspartic type dominating plant enzyme extract which is able to coagulate milk have been reported in milk thistle (*Silybum marianum* L. *Gaertn.*) (Vairo-Cavalli *et al*. 2005) [28]; artichoke flower (*Cynara scolymus* L.); *Onopordum turcicum* (Tamer 1993) [26]; rice kernels (Asakura *et al*. 1997) [3]. Thiol proteases, industrially popular as Cysteine proteases, have been reported in ginger rhizomes (*Zingiber* officinale) (Hashim *et al*. 2011) [12]; kiwifruits (*Actinidia chinensis*) (Katsaros *et al*. 2010) [14]; Ficin from *Ficus racemosa* (Devaraj *et al*. 2008) [6] and many more. Further, most of the plant based coagulase belongs to serine-type protease secreted in different plant parts in different concentrations. Neriifolin, a chymotrypsin-like serine protease, has been purified from the latex of *Euphorbia neriifolia* (Yadav *et al*. 2011) [29]; Streblin from *Streblus asper* (Tripathi *et al*. 2011) [27].

Proteases from practically every plant part, including seeds, flowers, and latex, have been isolated and researched for their efficacy as bovine milk coagulants. Various experiments comparing extracts from various parts of the same plant were conducted. Reported highest milk coagulating index with flower *Cynara Cardunculus* crude extract (Ben Amira *et al*., 2017) [4]. However, significant milk clotting activity was observed with crude extract of latex compared to stems, leaves, and flowers of *Calotropis gigantea* (Anusha *et al*., 2014) [1]. Another experiment with *Vallesia glabra* seed extract represented the maximum milk coagulation compared to other parts (González-Velázquez *et al*., 2021) [10]. Similarly, there have been reports indicating that pumpkin as a source of peptidase, exhibits a probability of having milk coagulation activity.

Raw milk contains proteases as part of its natural composition, and these enzymes play a role in the breakdown of proteins (Fox, 1989) [8]. The activity of these proteases can be influenced by various factors, including heat treatments used in the dairy industry (Leite *et al*., 2021) [16]. When raw milk is subjected to thermal processes like pasteurization or sterilization, may denature or inactivate the enzyme (Deeth *et al*., 2021) [5].

The present study emphasised the effect of pasteurization of milk on the coagulation efficiency of pumpkin seed-derived protease enzyme. This study also extends to the effect of temperature on the milk coagulation activity of pumpkin seed crude extract. This study also discussed in detail the extraction procedure of protease from pumpkin seeds. Therefore, the present study may report the efficacy of pumpkin's seed crude extract as a bovine milk peptidase along with the thermal effect on enzyme activity.

Materials and Methods

Ripened pumpkin fruit seeds were gathered to get milk coagulating peptidase. Pumpkin fruit were gathered from the local farmers in Karnal, Haryana, were processed into crude extract. The skim milk for this investigation was collected from Experimental Dairy, National Dairy Research Institute (NDRI), Karnal, India, while the chemicals were purchased from Hi Media.

Protease enzyme extraction from pumpkin seed

The matured pumpkin was cut properly and the respective seeds were gathered, cleaned with water, and dried for 2-3 days at ambient temperature. Thereafter, it was grounded into fine particle sized powder form. After that, the powdered seed was immersed for 48 hours at 4 °C in 0.01 M of buffer with a pH of 4.5. The whole mixture was then filtered through cheesecloth and centrifuged at 4010 g for 30 minutes while maintaining the temperature at 4 °C. Before doing additional experiments, the supernatant part was collected and kept at -20 °C.

Gelation of milk

The gelation property of the extract was evaluated by using skim milk as substrate. Seed extract (1 ml) was poured into the 10 ml skim milk substrate at experimental conditions to check the gelation.

Protein concentration determination

A technique defined by Lowry *et al*. (1951) [18] was used to ascertain the protein content of the pumpkin seed extract. Bovine serum albumin was used as the standard to create a standard curve. Following a 10-minute incubation period at room temperature, the various dilutions and sample solutions were mixed with an alkaline copper sulphate reagent. Following the addition of the reagent, the absorbance of each sample was noted at wavelength 660 nm using a spectrophotometer.

Milk coagulation activity (MCA)

The procedure described by Arima, Yu, and Iwasaki (1970) [2] was followed with minimal modifications in this study to test the milk clotting activity of the crude extract. For this analysis, the substrate was 10 millilitres of raw and pasteurised skimmed milk with a fat content of ≤0.5, pH- 6.6, and CaCl2 concentration of 0.01 M were taken. A temperature of 37 ºC was used to evaluate the clotting of milk after introducing 1 ml of crude seed extract into 10 ml of milk. As shown below, the milk clotting time was noted, and Equation (1) was used to compute the milk clotting activity (MCA).

$$\mathrm{MCA} = \frac{2400}{\mathrm{t}} \times \mathrm{d} \qquad (1)$$

Here,
MCA = Milk coagulation activity (U/ml)
t = Time
d = Dilution Factor

Temperature effect on MCA

The impact of variation in temperature on the MCA of the enzyme was determined by placing 10 mL of partially skimmed milk (0.5% fat, containing 0.01% CaCl2) in a hot water bath at 30, 35, 40, 45, 50 and 55 °C followed by addition of 1 mL of crude extract into it. The milk curdling time is defined as the time span between the addition of enzymes into milk and to time at which the first milk clot in milk is detected.

Proteolytic activity (PA) of seed extract

With only minor adjustments, the test procedure outlined by Sarath, Zeece, and Penheiter (2001) [21] was followed to ascertain the proteolytic activity of the seed extract. Using casein as a substrate, the proteolytic activity was measured.

Tris-HCl buffer (0.5 M, pH 6.8) was used to dissolve 1.5% casein. A mixture of 0.5 ml of the seed extract and 4.5 ml of dissolved casein was combined. The reaction was stopped by adding 5 ml of 10% TCA after the reaction had been incubated for 5 minutes at 54 °C. With the exception of the extract in the reaction mixture, the control was assumed to be the same. The reaction mixtures under test and control were let to stand for half an hour. Centrifugation was used to eliminate precipitates. A wavelength of 280 nm was used to measure the supernatant's absorbance.

Results and Discussion

Each ripe pumpkin fruit weighed about 1.5 kg, and the total weight of pumpkin seeds harvested was found to be between 60 and 70 grams. Different gelation initiation temperatures were discovered for both raw and pasteurised milk when the gelation activity was examined following the inoculation of pumpkin seed extract. The initial point for raw and pasteurised milk was found 30 and 37 ºC respectively.

Fig 1: Crude enzyme extract of pumpkin seed

Protein content of pumpkin

The crude enzymatic extracts obtained from pumpkin seed presented a protein content of around 3.167 mg /ml.

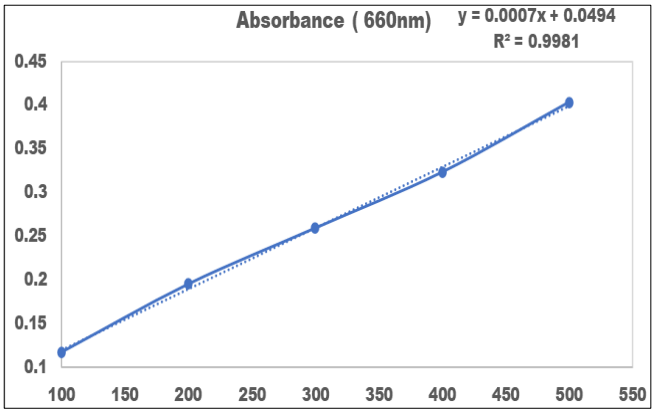

Fig 2: Standard graph of Bovine serum albumin

Milk coagulation activity (MCA)

The ability of the enzyme to hydrolyze κ-casein particularly is defined by its most significant characteristic, MCA. A milk clotting unit (MCU) is the volume of coagulant needed under certain conditions to coagulate 100 millilitres of milk in 40 min. In this investigation, the milk clotting activity of a crude pumpkin seed extract was found to be 151 U/ml for raw and 11.5 U/ml for pasteurized skimmed milk, respectively. The comparatively lesser value of MCA for pasteurized milk may be due to the inactivation of the native protease enzyme present in milk. Kumar *et al*. (2020) [15] also used a crude extract of pumpkin seed as a milk coagulant and reported an MCA of 104.3 U/ml.

Impact of temperature on Milk coagulation activity

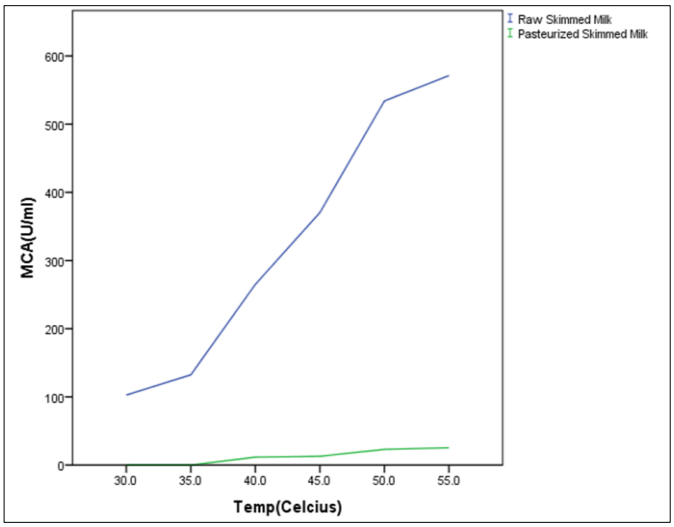

Fig 3: Impact of temperature on MCA

The effect of temperature on the MCA of crude extract of pumpkin seed is depicted in Fig. 3. The Observations showed that MCA increased with increase in temperature in the range of 30 to 55 °C. The higher temperature found the best temperature for this enzyme application in milk coagulation, which is similar to protease extracted from stems of *Wrightia tinctoria*, leaves and seeds of *Moringa oleifera Lam*, fruits like *Ficus johannis* and flowers like *Citrus aurantium*. (González-Velázquez *et al*., 2021) [10].

Proteolytic activity (PA)

The proteolytic activity of peptidases refers to the breakdowns of protein present in the curdled milk (mainly α_{s1}, α_{s2}, β, and κ-casein), which can develop bitter peptides in the long term. In the current investigation, the crude extract of pumpkin seed exhibited 0.776 U/ml proteolytic activity.

Conclusion

The findings of this study collectively suggest that pumpkin seed has the potential to be a milk coagulant. The Coagulant activity was observed over broad temperature range. The milk coagulation activity of crude pumpkin seed extract is significantly influenced by pasteurization of milk as well as variation in temperature. In future studies, to improve the understanding on the effect of pasteurization on MCA of crude extract of pumpkin seed, other parameters should also be examined: effect of variation in Ratio of dried seed powder to buffer used for enzyme extraction, effect of pH and concentration of enzyme on milk coagulation.

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