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Short Communication

Ixora coccinea flower extracts as a rapid test assay to confirm the proper pasteurization of milk

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Abstract

Pasteurization is crucial for hygienic safety and milk shelf-life extension. Alkaline phosphatase (ALP) activity determines pasteurization efficiency, but most methods are time-consuming or require sophisticated instrument facilities, which are rare in rural India dairy or collection centers. The study developed a simple, quick, and economical chromogenic test protocol based on the activity of Alkaline Phosphatase (ALP) in raw milk. The dye was prepared from *Ixora coccinea* water extract. Pasteurization inactivates ALP, resulting in a brownish green colour. The assay has a sensitivity of >0.5 units/L, allowing for easy visualization of pasteurized milk compared to raw milk.

Keywords: Alkaline phosphatase, Chromogenic dye, *Ixora coccinea*, Milk, Pasteurization

Highlights

- Traditional methods to detect pasteurization are time-consuming
- *Ixora Coccinea*-based chromogenic test detects pasteurization within 2 minutes
- Raw milk gives a colour change from brownish green to Olive-green
- Overcoming complexity due to a simple test suitable for resource-constrained dairy facilities

India is the world's largest producer of milk, accounting for nearly 21% of global milk production (FAO, 2021). Milk is a crucial source of macro and micronutrients, essential for growth and maintaining good health across all age groups.

Milk's perishable nature and high-quality nutrients make it an ideal medium for the growth of various microorganisms, potentially leading to spoilage or significant health risks (Lucey, 2015). Thermal treatment, primarily pasteurization, is a significant unit operation that increases or extend in milk's shelf life by getting rid of harmful and spoiling microorganisms. (Lewis, 1994; Holsinger *et al*., 1997). Alkaline Phosphatase (ALP) is a native enzyme, mainly found in milk fat globule membrane, used to indicate proper pasteurization of milk.

In order to ascertain whether milk has been properly pasteurized, FSSAI (2022) recommends the colorimetric method, despite its lengthy process time. Studies suggested that there are a few test protocols that were used to ascertain the proper pasteurization in milk, but the majority of these techniques required sophisticated instruments (fluorescence spectroscopy, ultraviolet spectroscopy, etc.) (Rankin *et al*., 2010). Therefore, adoption of these methods are not easy in dairy industry especially dairy industry situated in rural areas. As a result, a quick and simple test procedure is required to determine whether milk has been properly pasteurized. Researches have shown that synthetic chromogenic dyes can cause cancer and other harmful health effects (Gaikwad and Hakim, 2022). The application of these dyes in routine analysis must therefore be done with extra caution. As a result, using natural dyes for routine quality control analysis is growing in popularity.

In Ayurveda, *Ixora coccinea* (Rubiaceae) is referred to as Flame of the Woods or Jungle Geranium (Dontha *et al*., 2015). It is a typical Asian flowering shrub (Patil and Datar, 2015). *Ixora coccinea* is an evergreen, thick, multi-branched plant (Fig. 1). Flowers are produced continually throughout the year, ranging from 15 to 50, in many clusters on short, terminal panicle-like inflorescences that have two bracts that resemble leaves below (Deshpande *et al*., 2010). It has been reported that the extract of this flower has different therapeutic including anti-oxidant, anti-mitotic, anti-inflammatory, anti-tumor, hypoglycaemic, anti-microbial, anti-diarrhoeal and wound healing properties (Dontha *et al*., 2015). Deshpande *et al*. (2010) reported that this flower contains water-soluble pigment like anthocyanin, therefore, they are highly sensitive to acid-base titration. To the best of our

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knowledge, this flower-based dye has never been applied to ascertain the proper pasteurization of milk. Considering all the facts, based on ALP (activity) a rapid chromogenic test was developed to ascertain the pasteurization of milk using natural dye obtained from water extraction of *Ixora coccinea* (flower).

Fig. 1. Flowers of *Ixora coccinea*

Samples of pooled milk, in amber-colored glass bottles that were previously cleaned and sterilized, were collected from farmers of Amreli. Glass tubes holding three milliliters of milk sample were taken and heated under the following conditions: low-temperature long time (LTLT, 63ºC for 30 minutes), high-temperature short time (HTST, 75°C for 20 seconds) and boiled at 100ºC for 10 minutes in the water bath. Once the temperature reached the desired level, the tubes were taken out and immersed in ice water.

Fresh flowers were plucked from the Institute's Garden. Soon after plucking, flowers were washed under running water. Thereafter, 25.0 g of the flower petals were boiled for about 5 minutes; then it was diluted in 50 milliliters of double-distilled water, filtered through muslin cloth, and stored at a temperature of 6±2ºC in the refrigerator.

The substrate 4-Nitrophenyl disodium orthophosphate solution was prepared in a 1M tris buffer solution, with the pH adjusted to 8.2 using hydrochloric acid.

Two milliliters of buffer substrate and 0.8 milliliters of a water extract from *Ixora coccinea* (a natural dye) were added to a milliliter of milk in a dry, clean test tube. After 30 seconds of stirring, the mixture was incubated for two minutes at 37°C in a BOD incubator. The success of pasteurization was demonstrated by a change in colour. ALP activity of milk (cow and buffalo) was determined by the method described by FSSAI (2022).

All mammalian milk contains the ALP enzyme, which is typically connected to the membrane of the milk fat globule (Kosikowski, 1988). Studies suggested that there was noticeable variation in ALP concentration across various milk species (Sharma *et al*., 2014). In our nation, pasteurized milk from cow or buffalo milk is mixed and supplied by the milk industries. The ALP activity is reduced by effective pasteurization. Therefore, simple, rapid and in-expensive test protocol, to ascertain the proper pasteurization of milk, is a prime requirement for dairy industry especially in dairy industry situated in rural areas.

In this present study, the ALP activity of raw as well as heat treated milk was determined by FSSAI (2022) and result is depicted in Fig. 2. Significance ($P<0.05$) changes in ALP activity were observed among the raw and heat-treated milk samples (LTLT, HTST and boiled). It was observed (Fig. 2) that the ALP activity of raw milk was 1.043±0.000 U/mL, while the ALP activity of heat-treated (LTLT, HTST and boiled) milk were significantly ($P<0.05$) decreased during heating. According to earlier studies, properly pasteurizing or heating milk considerably decreased the amount of ALP enzyme present in milk (Prajapati *et al*., 2014). Dumitraşcu *et al*. (2015) reported that the α-helix and β-sheet of ALP were exposed to the alkaline phosphatase enzyme due to broken hydrogen bonds during pasteurization or boiling, causing ALP to lose its native structure.

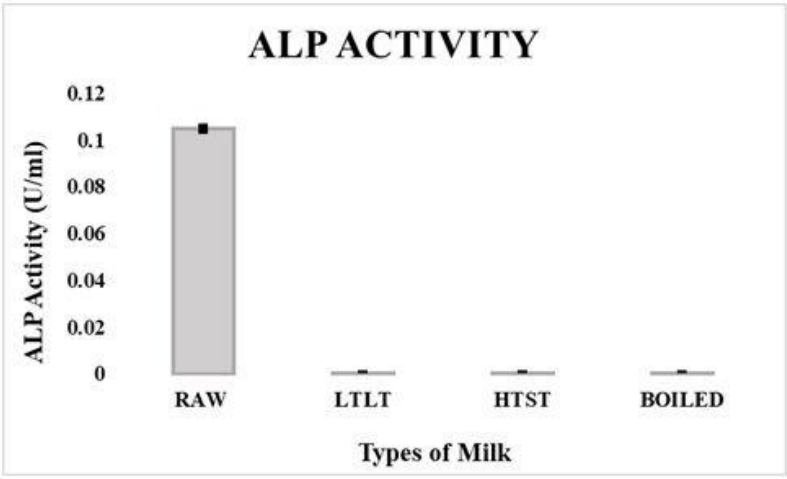

Fig. 2. Alkaline phosphatase Activity of raw and heat-treated milk

The ALP activities in raw milk (unheated) and heat-treated milk samples (LTLT, HTST, and boiled) were qualitatively measured by observing the colour change at the end of the developed protocol. It was also observed (Fig. 3) that raw milk from cows or buffaloes had activities of ALP enzymes; which were reduced after appropriate heat treatment (LTLT, HTST or boiling). In heat-treated (LTLT, HTST and boiled) milk samples (cow or buffalo), there was no activity of ALP enzyme, resulting in the formation of a brownish-green colour complex. Raw milk had an olive-green colour complex due to the ALP enzyme (Fig. 3). It can be assumed that the ALP of raw milk reacts with the substrate 4-nitrophenyl disodium orthophosphate and the natural chromogenic dye. The reaction changes the colour of raw or improperly pasteurized milk from brownish-grey to olive green within ten minutes of reaction time (Fig. 3). The assay has a sensitivity of >0.5 units/L with specificity of 100% (Table 1). Each sample was analysed in duplicate to determine true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results. The sensitivity and specificity of the developed test were found to be 100% (Table 1). This indicates that the assay is highly effective to ascertain proper pasteurization of milk. The developed test demonstrated exceptional specificity in accurately identifying the pasteurization of milk, as evidenced by the consistent detection.

Epidemiological studies indicated that the use of synthetic dyes could potentially cause serious diseases, even cancer (Kobylewski and Jacobson, 2012). Therefore, natural dyes obtained from various plants have been used in various food quality control applications. The ALP enzyme increased milk's alkalinity by hydrolyzing phosphate residues attached to casein, which were released in the liquid phase of the milk. Natural dyes derived from plants contain a number of bioactive components, including antho-cyanins, carotenoids, betalains, etc. (Molina *et al*., 2023). These bioactive components are very sensitive to pH; therefore, visible colour changes are usually observed due to pH changes (Gaikwad and Hakim, 2022). Therefore, natural dyes have been successfully used to determine the quality of various food products, such as meat, fish, fruits, etc. (Singh *et al*., 2018). However, to the best of our knowledge, there are no reports on the use of natural dyes to ensure proper pasteurization of milk. The flower extract was stored at refrigerator temperature (6±2°C) and the test was carried out at 2-day intervals to check the effectiveness. In this test, the effectiveness of the test remained the same for up to 30 days when kept at refrigerator temperature (6±2°C). The same test protocols were carried out with market milk samples (pasteurized) having different fat and SNF content. It was observed (Fig. 4) that the same efficiency resulted, although having different fat and SNF content of the

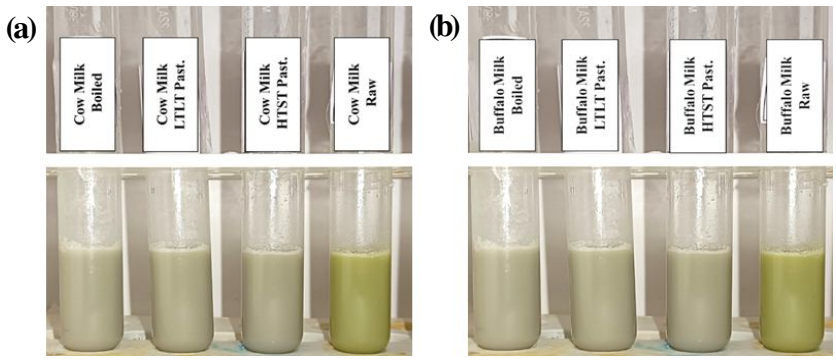

Fig. 3. Results of the chromogenic assay for raw and heat-treated milk (a) Cow Milk, (b) Buffalo Milk

Table 1. Sensitivity and specificity of the developed test

Sample	Positive	Sensitivity	Negative	Specificity
Raw milk	TP=10	Sensitivity	FP=0	Specificity
	FN= 0	=100× (10/10+0)	TN=10	=100× (10/10+0)
Pasteurized milk+	TP=10	=100× 1	FP= 0	=100× 1
Raw milk	FN= 0	=100%	TN=10	=100%
(0.5 µg/mL)				

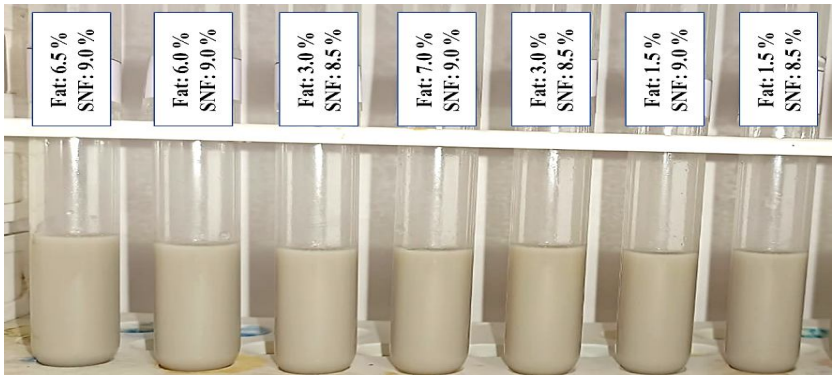

Fig. 4. Results of the chromogenic assay for milk samples of various brands (different fat and SNF content)

milk. Therefore, this quick and easy method can be carried out in any rural dairy, without any health risk. Hence, this Rapid indicative method can be utilized to accurately measure the activities of the enzyme ALP during milk pasteurization during processing.

The developed method is simple and able to distinguish between pasteurized and non-pasteurized milk. This developed protocol is easy, quick, affordable and sensitive for determining appropriate pasteurization of milk and easily adopted in every rural dairy industry.

Conflict of interest: The authors have no conflict of interest in this study.

Author's contribution: PAM: Tested the milk samples, wrote the first version of the article, performed chemical analysis of milk, meticulously collected and organized the data, and played a pivotal role in crafting the original draft of the article; TH: Provided in valuable expertise in designing the research plan, offered continuous guidance throughout the study, and conducted the final editing to ensure the article's quality; RS: Conducted thorough statistical analysis, curated the data with precision, and contributed significantly to the data-related aspects of the research; AS, RG: Played a key role in interpreting the data for the article, evaluated the entire study comprehensively and contributed to the overall quality of the research.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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