Effect of LP-system activation using externally added potassium thiocyanate, glucose oxidase, and glucose on total bacterial count and pH variability in raw buffalo milk

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DOI: https://doi.org/10.22271/chemi.2020.v8.i5c.10460

Abstract

Lactoperoxidase system has received high attention for raw milk preservation, because the system exerts hypo-thiocyanate ions to inhibit the growth of broad spectrum bacteria. The effectiveness of Lactoperoxidase system varies with LP-component concentration. LP-system was activated using different enzyme (glucose oxidase), substrate (glucose) concentrations in the presence of potassium thiocyanate at 37 °C. Comparison was made by measuring extent of bacterial multiplication (SPC) and pH variation. At 37 °C, for control milk, total bacterial count was around 5.057±0.057 log10 cfu/ml at 0 th hour but it showed increasing effect up to 8.732±0.024 log10 cfu/ml after 5 th hour. Whereas total bacterial count for LP-activated milk under varying concentrations of thiocyanate (30, 15 and 7.5 mg/l) with 2000 mg/l glucose and 400 U/l glucose oxidase was 7.239±0.239, 8.241±0.037 and 8.900±0.055 log10 cfu/ml respectively after 9 th hour at 37 °C that clearly indicated the effectiveness of LP-component concentration on bacterial multiplication. pH of activated milk was continuously decreasing in milk samples with lower enzyme (glucose oxidase) substrate (glucose) concentration.

Keywords: Lactoperoxidase system (LP), Buffalo milk, Standard plate count (SPC), Glucose oxidase (GO)

Introduction

Lactoperoxidase system (cold sterilization technique) has been reported to be useful (FAO/WHO, 1991 and 2005) [1, 2] for preservation of milk compared to the conventional methods. Enzyme lactoperoxidase catalyzed the oxidation of thiocyanate in the presence of hydrogen peroxide to activate a natural antimicrobial system in milk, the ‘Lactoperoxidase system’ which yields short lived oxidation products i.e. hypothiocyanate and higher oxyacids which act as antimicrobial agents. Hypothiocyanate ions (OSCN−) has a broad spectrum of antimicrobial effect against bacteria, fungi and viruses (Boots and Floris, 2006; Saad, 2008) [3, 4]. Lactoperoxidase system only active in the presence of three components: lactoperoxidase enzyme, thiocyanate (SCN−) and hydrogen peroxide. However, hydrogen peroxide (H2O2), is the only component of LP system, is normally not present in milk and necessitates extraneous addition to activate the system. (Elliott et al., 2004; Boots and Floris, 2006) [5, 6]. Lactoperoxidase system activation needs minimal quantities of H2O2 (8 mg/l) as compared to traditional method of milk preserving directly with H2O2 (800 mg/l) according to Fernandez et al. (2005) [6]. As described in the Codex Alimentarius Commission (CAC) guidelines: 14 mg of SCN− is added per litre of milk. The milk should then be mixed to ensure an even distribution of the SCN−. Plunging for about 1 minute with a clean plunger. Secondly, 30 mg of sodium percarbonate is added per litre of milk. The milk is then stirred for another 2–3 minutes to ensure that the sodium percarbonate is completely dissolved and the hydrogen peroxide is evenly distributed in the milk is normally satisfactory. According to FAO/WHO (2005) [2] this system is non-toxic when used according to the guidelines. It was proved that H2O2 up to 50 mg/l for the LP-system activation does not affect the milk components or nutritive value (Kumar and Mathur, 1989) [7]. Different sources of hydrogen peroxide are present as: liquid form (30% H2O2 solution), salt form (sodium carbonate peroxyhydrate, carbamide peroxide, sodium percarbonate, calcium and magnesium peroxides), microbial and enzymatically generated.
forms, where in enzyme form mainly glucose oxidase, xanthine oxidase, lactose oxidase and hexose oxidase are used to generate \( \text{H}_2\text{O}_2 \) (Luck, 1962; Seifu et al., 2005; Willrett et al., 2011; Lara and Alcain, 2019) [8, 9, 10, 11].

Different uses of enzymes for the production of environment friendly pure compounds are the emerging approaches of biotechnology. De jong et al. (1993) [12] reported that enzymatic production of hydrogen peroxide is preferred because it is possible to add both the substrate and enzymes (especially food grade oxidoreductases) extraneously in the system. Feng Xu (2005) [13] reported that among these enzymes, major commercial/industrial oxidase which is extensively used for baking, dairy and other food applications is glucose oxidase. Hence, the present work was proposed to explore the possibilities of glucose oxidase in combination with glucose to generate hydrogen peroxide and to study its applicability in the activation of the LP system using in raw buffalo milk.

**Materials and Methods**

Fresh pooled buffalo (Murrah) milk sample were collected from Livestock research centre, National Dairy Research Institute, Karnal, India. The enzyme used was *Aspergillus niger* glucose oxidase (100 U/mg) and potassium thiocyanate was procured from s.d. Fine- chem Ltd., Mumbai, India. Glucose and plate count agar was purchased from Hi-media, Pvt. Ltd. Mumbai.

**LP-system activation**

One liter fresh pooled buffalo (murrah) milk was taken in wide mouth bottles, having different glucose oxidase (400 units), glucose (500, 1000, 2000 mg) and potassium thiocyanate (7.5, 15 and 30 mg) concentrations as and placed the samples in B.O.D incubator at 37 °C ± 1 °C. Milk samples were drawn after 6, 12, 24, 48 h. This bacterial count from 7.73 log10 cfu/ml to 14.2 log10 cfu/ml after 5 h was reported. Abdallah (2005) [8, 9, 10, 11] reported that among these enzymes, major commercial/industrial oxidase which is extensively used for baking, dairy and other food applications is glucose oxidase. Hence, the present work was proposed to explore the possibilities of glucose oxidase in combination with glucose to generate hydrogen peroxide and to study its applicability in the activation of the LP system using in raw buffalo milk.

**Standard plate count**

Microbial quality of raw milk was assessed generally for total plate count and pH variation in activated system.

**Effect LP-activation on total plate count**

The data presented in table 1 depicted the effect of different concentrations of potassium thiocyanate i.e. 7.5 mg/l, 15 mg/l and 30 mg/l and glucose 500 mg/l, 1000 mg/l and 2000 mg/l in combination with 400 U/l of glucose oxidase for LP-system activation. The resulted total bacterial count after LP-system activation was 4.65±0.048, 5.078±0.036, 6.230±0.026 and 7.239±0.239 and 5.743±0.028, 5.971±0.016, 6.241±0.037 and 7.866±0.006 log10 cfu/ml when LP-components used were 30 mg potassium thiocyanate, 400 U/l glucose oxidase in combination with 2000 and 1000 mg/l glucose at 3rd, 5th, 7th and 9th hour respectively after activation. Whereas higher bacterial counts has been detected for 15 mg and 7.5 mg thiocyanate treatment i.e. 8.241±0.037 and 8.900±0.055 log10 cfu/ml respectively at 9th hour of activation. 8.732±0.024 log10 cfu/ml has been detected after 5th hour of incubation at 37°C in untreated milk. These results are in line with the findings of Villa et al. (2014) [15] who reported that LP-system activation in fresh milk resulted in reduction of total bacterial count from 7.73 log10 cfu/ml to 7.5 cfu/ml. This bacterial growth reduction phenomenon could be explained that total bacterial count is in line with the number of sulphhydril groups that should be oxidized by OSCN ions. According to Bjorck et al. (1975) [16] bactericidal effect increased with the increase in thiocyanate concentration from 0.085 to 0.175 mM respectively against *P. fluorescens*. Abdallah (2005) [17] worked with 0.017 g/l potassium thiocyanate and 0.034 g/l sodium percarbonate and observed that growth (TVC) was suppressed to 4x10⁶ cfu/ml at 10th hour, since the control samples crossed this level in 4th hour at 30°C. As the thiocyanate (NaSCN) and hydrogen peroxide (sodium percarbonate) ratio increased (10:10, 20:20, 30:30) there was significant log reduction observed for total viable count (TVC) i.e. log 5.34 cfu/ml (30:30), log 5.58 cfu/ml (20:20) and log 6.04 cfu/ml (10:10) after 10 h compared to control samples, reached to unacceptable level (log 6.12 cfu/ml) after 6 h at 40°C (Masud et al., 2010) [18].
The bacterial count was lesser only those with a higher concentration of thiocyanate in milk. The value of untreated milk was 6.06±0.038 to 6.39±0.018 up to 10 h. Table 1 shows the effect of this concentration has been screened for pH of milk activated milk. The total plate count of control and untreated milk was continuously above 6.67±0.006. This pH value suppression was in agreement with total bacterial count. The LP-component concentrations, where bacterial count was lesser only those concentrations maintained the pH value to longer period. Similar findings were observed by Masud et al. (2010) who reported that three different ratios of NaSCN and sodium percarbonate as 10:10, 20:20, and 30:30 were used for activation of LP-system. They found that pH of activated milk was 6.26 (10:10), 6.34 (20:20) after 10 h and 6.30 (30:30) after 16 h but for control milk samples pH (6.20) was crossed after 6 h at 40°C that clearly indicate the increased stability of raw milk.

Table 1: Effect of LP-activation on microbial stability of raw buffalo milk

| Glucose (mg) | Potassium thiocyanate (mg) | 0      | 3      | 5      | 7      | 9      |
|-------------|---------------------------|--------|--------|--------|--------|--------|
| 2000        | 30                        | 5.057±0.057<sup>AB</sup> | 4.651±0.048<sup>Ba</sup> | 5.078±0.036<sup>Ca</sup> | 6.230±0.026<sup>Da</sup> | 7.239±0.239<sup>Ea</sup> |
|             | 15                        | 5.057±0.057<sup>AB</sup> | 5.060±0.019<sup>AB</sup> | 5.332±0.010<sup>Ba</sup> | 6.462±0.015<sup>Ba</sup> | 8.241±0.037<sup>Ea</sup> |
|             | 7.5                       | 5.057±0.057<sup>AB</sup> | 5.161±0.015<sup>Ba</sup> | 6.543±0.025<sup>Ca</sup> | 6.648±0.015<sup>Ca</sup> | 8.900±0.055<sup>Ea</sup> |
| 1000        | 30                        | 5.057±0.057<sup>AB</sup> | 5.743±0.028<sup>Ba</sup> | 5.971±0.016<sup>Ba</sup> | 6.241±0.037<sup>Ba</sup> | 7.886±0.006<sup>Ea</sup> |
|             | 15                        | 5.057±0.057<sup>AB</sup> | 5.929±0.005<sup>Ba</sup> | 6.290±0.011<sup>Ba</sup> | 6.421±0.041<sup>Ba</sup> | Clot |
|             | 7.5                       | 5.057±0.057<sup>AB</sup> | 6.060±0.015<sup>Ba</sup> | 6.550±0.006<sup>Cb</sup> | 7.066±0.013<sup>Cb</sup> | Clot |
| 500         | 30                        | 5.057±0.057<sup>AB</sup> | 5.949±0.005<sup>Ba</sup> | 6.109±0.007<sup>Ca</sup> | 6.849±0.151<sup>Cb</sup> | 9.468±0.004<sup>Ea</sup> |
|             | 15                        | 5.057±0.057<sup>AB</sup> | 5.961±0.007<sup>Ba</sup> | 6.352±0.010<sup>Cb</sup> | 7.406±0.026<sup>Cb</sup> | Clot |
|             | 7.5                       | 5.057±0.057<sup>AB</sup> | 6.017±0.008<sup>Ba</sup> | 6.803±0.003<sup>Cb</sup> | 8.102±0.009<sup>Cb</sup> | Clot |
| Control     |                           | 5.057±0.057<sup>AB</sup> | 5.690±0.486<sup>Bb</sup> | 8.732±0.024<sup>Cb</sup> | Clot | Clot |

*Glucose oxidase concentration 400 U/l
Values are mean ± standard error of three replicates
<sup>ab</sup> Mean<sup>AB</sup> Means in a column with same superscript are not significantly different (p>0.05)
<sup>ab</sup> Means in a rows with same superscript are not significantly different (p>0.05)

Effect LP-activation on pH of milk

It has been clearly shown from table 1 that out of three different potassium thiocyanate concentrations 30 mg/l has shown maximum effect on bacterial reduction, so therefore the effect of this concentration has been screened for pH of treated and untreated milk using different concentrations of glucose oxidase i.e. 200, 400, 600, 800 and 1000 U/l and glucose i.e. 500, 1000, 2000 mg/l. Figure 1 showed the effect of LP-system on pH of milk until 10<sup>th</sup> hour after activation at 37°C. Since after 5<sup>th</sup> hour the critical value of untreated milk was reached. As can be seen in figure 1 that LP-activation period was able to maintain the pH of milk into the range from 6.06±0.038 to 6.39±0.018 until 10<sup>th</sup> hour, while for untreated milk pH decreased to 6.315±0.072 after 5<sup>th</sup> hour and 4.935±0.026 at 10<sup>th</sup> hour of incubation at 37°C. Prior to activation treatment, fresh milk samples were resulted to pH value of 6.67±0.006. This pH value suppression was in agreement with total bacterial count. The LP-component concentrations, where bacterial count was lesser only those concentrations maintained the pH value to longer period. Similar findings were observed by Masud et al. (2010) who reported that three different ratios of NaSCN and sodium percarbonate as 10:10, 20:20, and 30:30 was used for activation of LP-system. They found that pH of activated milk was 6.26 (10:10), 6.34 (20:20) after 10 h and 6.34 (30:30) after 16 h but for control milk same pH (6.20) was crossed after 6 h at 40°C that clearly indicate the increased stability of raw milk.

Fig 1: Effect of LP-system activated using glucose oxidase, glucose and potassium thiocyanate (30 mg/l) on pH of milk

Conclusion

Varying concentrations of potassium thiocyanate i.e. 7.5 mg/l, 15 mg/l and 30 mg/l and glucose 500 mg/l, 1000 mg/l and 2000 mg/l in combination with 400 U/l of glucose oxidase when used for LP-system activation. The total plate count of activated milk using different concentration of thiocyanate in combination with 2000 mg glucose and 400 U glucose oxidase was 7.239±0.239, 8.241±0.037 and 8.900±0.055 respectively after 9<sup>th</sup> hour at 37°C that clearly indicate the effect of LP-component concentration on bacterial multiplication. pH of activated milk was continuously decreasing in milk samples with lower enzyme (glucose oxidase) substrate (glucose) concentration.

Acknowledgement: Funds used for this study were Grants from NDRI, Karnal.

References

1. FAO/WHO. Guidelines for the preservation of raw milk by use of the lactoperoxidase system CAC/GL 13-1991.
2. FAO/WHO Benefits and Potential Risks of the Lactoperoxidase system of Raw Milk Preservation.
3. Boots JW, Floris R. Lactoperoxidase: From catalytic mechanism to practical applications. International Dairy Journal. 2006; 16:1272-1276.

4. Saad AH. Activation of milk lactoperoxidase system for controlling pseudomonas in cow’s milk. International Journal of Dairy Science. 2008; 3:131-136.

5. Elliot RM, McLay JC, Kennedy MJ, Simmonds RS. Inhibition of food born bacteria by lactoperoxidase system. International Journal of Food Microbiology. 2004; 91:73-81.

6. Fernandez O, Marrero E, Capdevila JZ. Safety considerations on lactoperoxidase system use for milk preservation. Revista de Salud Animal. 2005; 27(3):186-189.

7. Kumar S, Mathur BN. Incidence of LP-System on the nutritional quality of milk proteins. Indian Journal of Dairy Science. 1989; 42:198-202.

8. Luck H. The use of hydrogen peroxide in milk and dairy products. In milk hygiene: Hygiene in milk production, processing and distribution; World health Organization Monograph Series No.48. World Health Organization: Geneva, 1962, 423-447.

9. Seifu E, Buys EM, Donkin EF. Significance of lactoperoxidase system in dairy industry and its potential application: A review. Trends Food Science and Technology. 2005; 16:137-154.

10. Willrett D, Kersti H, Abdul F, Gregory S. Preservation of Raw Milk in Developing Countries by Addition of Hexose Oxidase to Activate the Lactoperoxidase System (Abstract), 2011.

11. Lara AS, Alcaine SD. Lactose oxidase: A novel activator of the lactoperoxidase system in milk for improved shelf life. Journal of Dairy Science. 2019; 102(3):1933-1942.

12. De Jong SJ, De Haan BR, Tan HS. Long term antimicrobial activity obtained by sustained release of hydrogen peroxide (US patent). Ser. No. 07/969,153, 1993.

13. Feng Xu. Application of oxidoreductases: Recent progress (A Review). Industrial biotechnology, 2005, 38-50.

14. IS: 1479. Methods of test for dairy industry. Part III. Bacteriological analysis of milk, 1977.

15. Villa VY, Legowo AM, Bintoro VP, Al-Baari AN. Quality of fresh bovine milk after addition of hypotiocyanate –rich- solution from lactoperoxidase system. International Journal of Dairy Science. 2014; 9(1):24-31.

16. Björck L, Rosen CG, Marshall V, Reiter B. Antibacterial activity of the lactoperoxidase system in milk against Pseudomonads and other gram-negative bacteria. Applied Microbiology and Biotechnology. 1975; 30:199-204.

17. Abdallah MIM. A new trial for preservation of raw cow milk at 30 °C and 5 °C by activation of the natural Lactoperoxidase System. The 4th International Science Conference. The Role of Veterinary Medicine for Community, Application of Biotechnology in Veterinary Medicine. 5-6 April 2005, Mansoura, Egypt, 2005.

18. Masud Tariq, Khalid S, Maqsood S, Bilal A. Preservation of raw buffalo’s milk by the activation of lactoperoxidase system and its effect on yogurt preparation. Journal of Food Processing and Preservation. 2010; 34:241–254.