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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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Abstract
Lactoperoxidase system has received high attention for raw milk preservation, because the system exerts hypothiocyanate 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 0th hour but it showed increasing effect up to 8.732±0.024 log10 cfu/ml after 5th 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 9th 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 (H_2O_2), is the only component of LP system, is normally not present in milk and necessitates extraneous addition to activate the system. (Elliot *et al.*, 2004; Boots and Floris, 2006) [5, 3]. LP- system activation needs minimal quantities of H_2O_2 (8 mg/l) as compared to traditional method of milk preserving directly with H_2O_2 (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 H_2O_2 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% H_2O_2 solution), salt form (sodium carbonate peroxihydrate, 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 H_2O_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 0th, 3rd, 5th, 7th and 9th hour every one hour interval for determination of standard plate count and pH variation in activated system.

Standard plate count

Microbial quality of raw milk was assessed generally for total plate count at 37°C for 24-48 h. This method is highly effective for the determination of total number of viable bacteria in milk as described in IS: 1479 Part III (1977). Immediately before sampling, the contents were vigorously mixed until thorough mixing was assured. 1.0 ml of milk sample was aseptically transferred in 9.0 ml normal saline (0.45% NaCl) and mixed the content homogenously. This was 1st dilution. 1.0 ml from 1st dilution was transferred into 9.0 ml normal saline in test tube and mixed the content. This was the second dilution. Decimal dilutions up to 10^{-6} using sterile 9.0 ml dilution blank were prepared. 1.0 ml of each decimal dilution (10^{-4}, 10^{-5}, and 10^{-6}) was transferred into three sterile petri dishes.12-15 ml melted and cooled sterile nutrient agar (40-45°C) was then transferred to each petri dish. The contents were mixed by rotating petri dishes clock-wise and anti-clock wise and allowed to solidify on a level surface. Petridishes were incubated in inverted conditions at 37 ^{0}C for 24-48 h. Colonies counted on petridishes should not be more than 300 and results were recorded.

pH analysis

EUTECH pH Tutor was used for the analysis of pH variation after LP-system activation.

Statistical analysis

Experiments were performed in triplicates and the data expressed as mean value with standard deviation. Analysis of variance was performed using IBM SPSS Statistics software V21.0 to establish relationship between LP-system raw buffalo milk as affected by enzyme substrate concentration and reaction time using significant difference of 0.05. Independent variables included enzyme, substrate concentrations and reaction time as well as concentrations and reaction time interactions. Means and standard deviations were calculated, when *F*-values were significant ($P< 0.05$), mean differences were separated by the least significant difference procedure.

Results and Discussion

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 4.651±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 7.886±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 sulfhydryl 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 $4x10^6$ 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].

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

LP-Components		Standard Plate count (log10 cfu/ml) Storage period (h)				
Glucose (mg)	**Potassium thiocyanate (mg)**	**0**	**3**	**5**	**7**	**9**
2000	30	5.057 ± 0.057^{Aa}	4.651 ± 0.048^{Ba}	5.078 ± 0.036^{Ca}	6.230 ± 0.026^{Da}	7.239 ± 0.239^{Ea}
	15	5.057 ± 0.057^{Aa}	5.060 ± 0.019^{Bb}	5.332 ± 0.010^{Cb}	6.462 ± 0.015^{Db}	8.241 ± 0.037^{Eb}
	7.5	5.057 ± 0.057^{Aa}	5.161 ± 0.015^{Bc}	6.543 ± 0.025^{Cc}	6.648 ± 0.015^{Dc}	8.900 ± 0.055^{Ec}
1000	30	5.057 ± 0.057^{Aa}	5.743 ± 0.028^{Bb}	5.971 ± 0.016^{Cb}	6.241 ± 0.037^{Db}	7.886 ± 0.006^{Eb}
	15	5.057 ± 0.057^{Aa}	5.929 ± 0.005^{Be}	6.290 ± 0.011^{Ce}	6.421 ± 0.041^{De}	Clot
	7.5	5.057 ± 0.057^{Aa}	6.060 ± 0.015^{Bf}	6.550 ± 0.006^{Cf}	7.066 ± 0.013^{Df}	Clot
500	30	5.057 ± 0.057^{Aa}	5.949 ± 0.005^{Bd}	6.109 ± 0.067^{Cd}	6.849 ± 0.151^{Dd}	9.468 ± 0.004^{Ed}
	15	5.057 ± 0.057^{Aa}	5.961 ± 0.007^{Bf}	6.352 ± 0.010^{Cf}	7.406 ± 0.026^{Df}	Clot
	7.5	5.057 ± 0.057^{Aa}	6.017 ± 0.008^{Bg}	6.803 ± 0.003^{Cg}	8.102 ± 0.009^{Dg}	Clot
Control		5.057 ± 0.057^{Aa}	5.690 ± 0.486^{Bh}	8.732 ± 0.024^{Ch}	Clot	Clot

*Glucose oxidase concentration 400 U/l
Values are mean ± standard error of three replicates
[ab] Mean [AB] Means in a column with same superscript are not significantly different (p>0.05)
[ab] 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 upto 10th hour after activation at 37˚C. Since after 5th 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 upto 10th hour, while for untreated milk pH decreased to 6.315±0.072 after 5th hour and 4.935±0.026 at 10th 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) [18] 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.

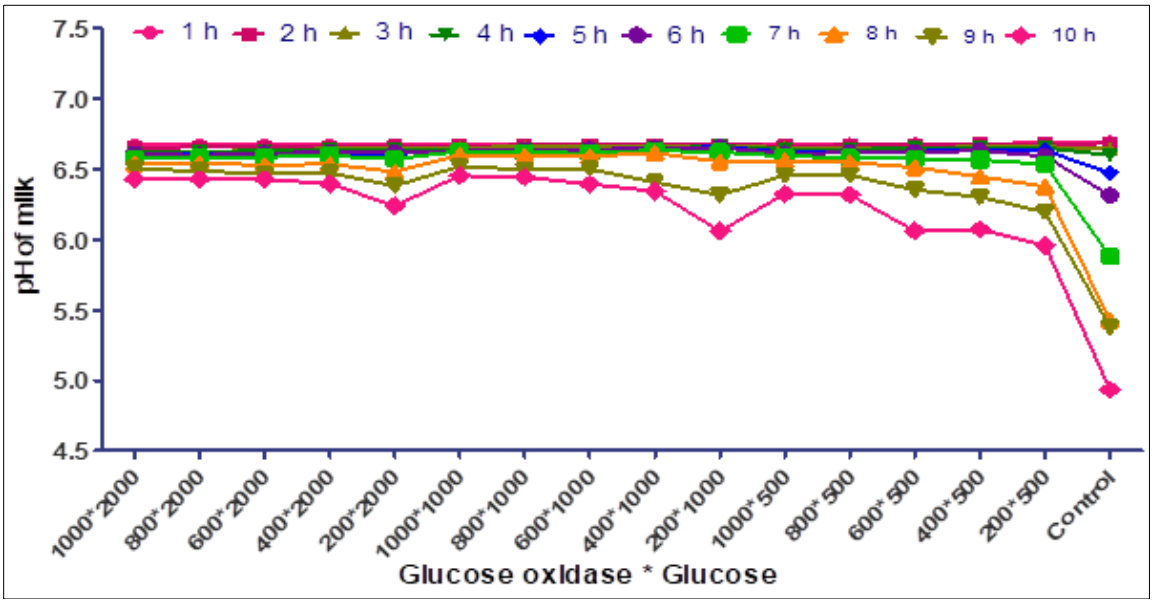

Fig 1: Effect of LP-system activated using glucose oxidase, glucose and potassium thiocyanate (30 mg/l) on PH of milk

Conclusion

Varing 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 9th 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.

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