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Development of one step spot detection method for hydrogen peroxide in raw milk as preservative and adulterant

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Abstract— *There was an incident in our locality that one-ton litre raw milk was detained in check post alleging adulteration of milk with Hydrogen peroxide. The news was reported in media and widely discussed by public. In the view of incident five under graduate students under my guidance conducted study of effect of hydrogen peroxide in raw milk. The study includes the preservative and adulterant characteristics of hydrogen peroxide in raw milk. They developed a simple method for quick detection of hydrogen peroxide in the raw milk. The investigation proved that optimum amount of hydrogen peroxide can act as preservative particularly in the occasion when quick cooling of milk is not possible. Addition of hydrogen peroxide increases shelf-life anti-bacterial properties of the row milk. The study developed a one step spot detection method for hydrogen peroxide in row milk. The test gives positive result above*

Keywords— ***Raw milk, adulterant, hydrogen peroxide, Methylene blue, Anti-microbial, Lactoperoxidase, Clot on boiling***

I. INTRODUCTION

Milk is one of the most complete foods for humans, containing nutrients including proteins, minerals, fats, carbohydrates, and vitamins, and is widely marketed and consumed by the population across the globe. The composition of milk is the combination of several solid components (12–13%) in water. These components, their distributions, and interactions determine the structure and functional properties of the milk, together with its suitability for processing and consumption. This rich composition makes milk an excellent substrate for the growth of various groups of microorganisms, hence storage and transportation of milk is always challenging [1]. Currently refrigeration is the only permitted method of preservation of milk. But it is not a viable method in rural, underdeveloped and conflict region due to the lack of electricity and high cost. It is observed that raw milk is deteriorated within two or three days even after storing in refrigerator [2]. Hence the practice of a relatively harmless preservative method is advisable. The components added for the extension of shelf life shall be nontoxic, non-reactive with components of milk and inexpensive.

Milk has limited inherent ability to inhibit microbial growth due to the presence of molecules Lactoperoxidase(LP), Hydrogen Peroxidise H_2O_2 in the milk. The average concentration of LP in row milk is 39mg/L. This enzyme molecule with Hydrogen Peroxide together activates the peroxidation of thiocyanate to hypothiocyanite ion, which is supposed as the main antimicrobial component of milk. The challenging factor of this lactoperoxidase system of protection is insufficient concentration of Hydrogen Peroxide. Hence the moderate addition of Hydrogen peroxide in the milk will enhance the antimicrobial property of the row milk [3].

Hydrogen peroxide is one of the most versatile chemicals. It is an environmentally-friendly oxidant that

is widely employed in foods, textiles, and personal care products. United States accepted hydrogen peroxide for the production of cheeses up to maximum 0.05% of the weight of the milk. It has been recommended as a dairy preservative in tropical countries [4]. The side effect of hydrogen peroxide will be suppressed by the presence lactoperoxidase and thiocyante ions present in the milk [5]. The use of liquid and vapor phase H_20_2 is common in the food, pharmaceutical and medical industries to reduce or eliminate bacterial contamination. Hydrogen Peroxide control of microbial deterioration of fruits and vegetables are common. The wide application of Hydrogen Peroxide in food industry is due to its broad-spectrum activity as well as its nontoxic nature following degradation [6]. The addition of hydrogen peroxide beyond the optimum level has been strictly banned. Over concertation of hydrogen peroxide in row milk decreases its nutritional value. It may lead to the oxidative destruction of vitamins and other components.

There may chances of addition of hydrogen peroxide to halt the microbial activity in raw milk close to the expiry date or already unsuitable for the consumption. Hence the monitoring of concentration of hydrogen peroxide in the milk is essential. There are a few complicated analytical techniques to detect and quantify presence of hydrogen peroxide in the milk. Most of them are highly sensitive, requires prior sample preparations. It required trained operators and costly instruments. Hence simple and quick methode of detection of hydrogen peroxide workable with un educated, rural people are vital [7-9]. This paper discusses a simple method of detection of hydrogen peroxide in the raw milk. It also studies optimum concentrations of hydrogen peroxide in the milk for better shelf life without compromising the quality of milk.

II. MATERIALS AND METHODS

Fresh Cow milk samples were collected directly from farmers in clean, sterilized bottle from the rural areas of Kozhikode, Kerala, India. All samples were collected in the morning, the day experiments started. Collected samples kept in refrigerator till experiments begin. Hydrogen Peroxide (diluted in the ratio 1: 2.5 with distilled water), Methylene Blue, Sodium stearate, KI were purchased from Merck (Germany). Mineral salt broth and nutrient agar are obtained from Himedia Chemicals (India). All solution were prepared in sterilized water.

2.1 Preparation of milk sample:

Five different milk samples were prepared by mixing 100ml of raw milk with different concentrations of hydrogen peroxide. Sample A, without hydrogen peroxide was considered as blank sample. All samples were kept in room temperature and all test were carried out in room temperature.

Table 1: Milk sample

SAMPLE	% of H_2O_2 (V/V)
A	0
B	0.05
C	0.1
D	0.15
E	0.2

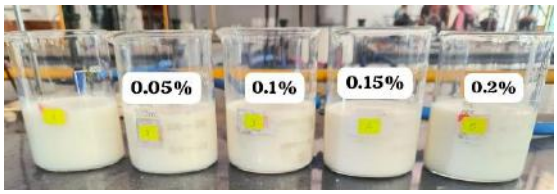


Image-1: Milk samples

2.2 Changes in flavour:

Appearance of unpleasant (slight sour or bitter) flavour was detected by organoleptic test periodically and tabulated.

2.3 Clot on boiling (COB):

5mLof milk taken in clean test tube from each sample and boiled over Bunsen burner in different time intervals. Clotting or coagulation of the milk samples was tested and tabulated.

2.4 Acidity test:

10mL from each milk sample was taken and diluted with equal amount of water. 3 to 4 drops phenolphthalein was added and shaken well. The mixture was titrated against 0.1N NaOH solution till the colour changed to pink. Noted the burette reading and percentage of acidity was calculated.

% acidity =(Volume of NaOH × 0.1 × 90 × 100)/ (V×1000)

2.5 Methylene blue reduction (MBR) test:

Previously prepared 1 ml of 1ppm Methylene Blue solution added to 10ml of milk taken in separate beakers corresponding to each sample and kept in room temperature and noted the colour change.

2.6 Anti-Bacterial analysis

Anti-bacterial analysis was carried out using Colony forming Unit (CFU) method. Each milk sample was

serially diluted to seven times using diluent phosphate Buffer. 1 ml of diluted samples were added to Petry plates contain Agar media at 45^0C. All plates were incubated for three days and bacterial count was recorded.

2.6 One step spot detection of H_2O_2

Previously prepared 4% Potassium iodide solution and 10ml of sodium stearate solution taken in dropper. The mixture added to the milk samples containing hydrogen peroxide and noted the changes.

III. RESULT AND DISCUSSIONS

3.1 Changes in flavour:

The trend of change in flavour of different samples of milk when kept in room temperature given in table 2. The milk sample without hydrogen peroxide exhibited change in flavour at earliest. The sample containing 0.2% hydrogen peroxide can withstand in room temperature over night without changing the flavour. It results explicitly proved that hydrogen peroxide (H_2O_2) has significant role in preserving flavour of milk [10]. Flavour of milk depends upon stability of milk constituents such as lactose, lipids, citrate and proteins, the stability of milk constituents and flavour depend temperature, p^H, water content etc [11]. Change in flavour of milk is attributed to the formation of various undesirable compounds in the milk. Lactose will be converted to furans, pyrones, cyclopentanes, carbonyl compounds etc. similarly, lipids will be converted to methyl ketones, lactones, and aldehydes [12]. The formation of undesirable compounds depends upon physico- chemical conditions of the milk sample. The presence of hydrogen peroxide in the milk has been arrested all changes in the milk and preserved the physio chemical balance of the milk in the room temperature. It is due to the presence of reactive oxygen generated by hydrogen peroxide, which inhibit microbial growth and prevent oxidative changes of milk sample.

Table 2: Changes in flavour

Hour	A	B	C	D	E
1	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
2	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
3	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
4	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
5	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
6	Pleasing	Pleasing	Pleasing	Pleasing	Pleasing
16	Sour	Sour	Slightly sour	Pleasing	Pleasing
17	Sour	Sour	Sour	Pleasing	Pleasing
18	Sour	Sour	Sour	Slightly sour	Pleasing
19	Bitter	Bitter	Sour	Sour	Pleasing
20	Bitter	Bitter	Bitter	Sour	Pleasing

3.2 Clot on boiling:

The clotting behaviour of milk samples given table3. The sample without hydrogen peroxide exhibited clot on boiling at 9.5hrs whereas sample E didn't exhibit clot on boiling even after keeping overnight at room temperature. The result is matching with the observations of flavour change. The result of clot on boiling showed the ability of hydrogen peroxide to preserve the milk sample at room temperature. Clotting of milk taking place due to the enzymatic actions of various bacteria and fungus present in the milk [13]. The clotting of milk produces annoying chemicals and render milk unfit for use [14]. The presence of hydrogen peroxide inhibits the growth microorganisms in the milk hence prevent from clotting of milk while storing in room temperature. The reactive oxygen generated by hydrogen peroxide prevent the microbial growth the extend the shelf life of the sample. Clotting of milk is largely associated with flavour change, the presence of hydrogen peroxide has the collective effect on flavour change and clotting.

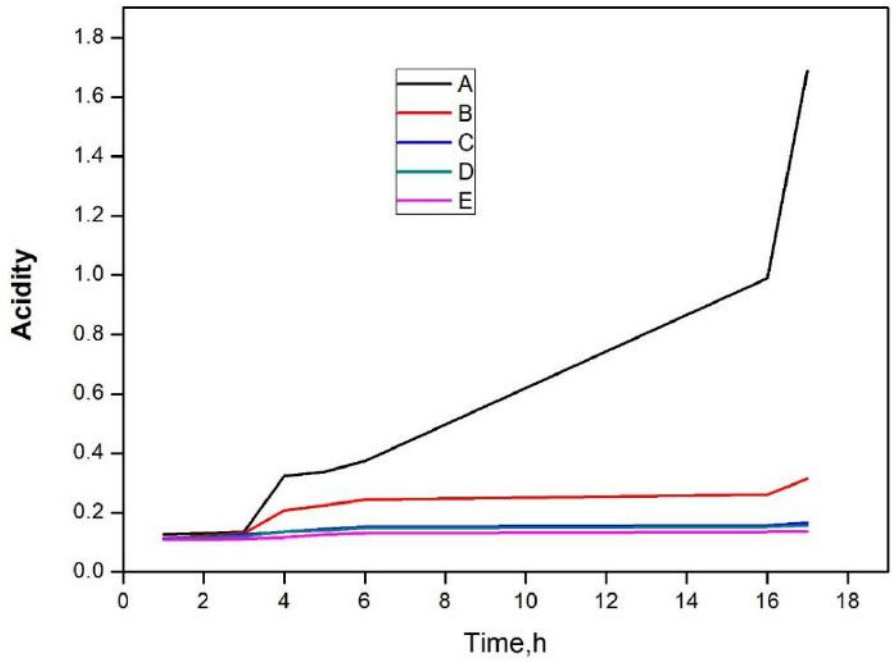
3.3Acidity

Titratable Acidity of the milk is its capacity of neutralisation with the base [15]. It is a very important parameter for the technical evaluation of the quality of milk. The titratable acidity of samples in different time intervals were recorded and given in table 4. The different components of the milk are acidic and contribute to normal acidity value. These components are carbon dioxide,

protein, phosphate and citrate. When bacterial count increases, lactose converted to lactic acid and increased the titratable acidity of the sample. the titratable acidity of fresh milk typically varies from 0.15 to 0.20 depending on the composition of milk [16]. The table shows that sample without hydrogen peroxide showed significant change in acidity within in 20hrs. it has exhibited gradual increase of acidity from 0.126 to 1.6875 where as such a gradual change was not observed in other samples containing hydrogen peroxide. The sample E containing maximum hydrogen peroxide showed lowest change in acidity. It clearly correlates the bacterial growth in the milk samples. When hydrogen peroxide is added to milk, it can potentially lower the overall acidity of the milk due to its antimicrobial property. Hydrogen peroxide added in the either kill or inhibiting the growth of bacteria that contribute to milk acidity through fermentation. By reducing the population of bacteria, the production of lactic acid through fermentation is decreased, leading to lower acidity in the milk. Hydrogen peroxide would also react with proteins in milk, potentially stabilizing them and preventing their degradation over time. Since proteins can act as buffers, helping to regulate the pH of the milk. By stabilizing proteins, hydrogen peroxide may indirectly contribute to maintaining a lower acidity level in the milk.

Table 3- clotting behaviour of milk

Hour	A	B	C	D	E
1	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
2	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
3	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
4	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
5	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
6	Free flowing	Free flowing	Free flowing	Free flowing	Free flowing
16	Clotted	Slightly Clotted	Free flowing	Free flowing	Free flowing
17	Clotted	Slightly Clotted	Free flowing	Free flowing	Free flowing
18	Clotted	Clotted	Free flowing	Free flowing	Free flowing
19	Clotted	Clotted	Slightly Clotted	Free flowing	Free flowing
20	Clotted	Clotted	Clotted	Slightly clotted	Free flowing



Graph -1: Titratable Acidity

3.4 Methylene blue Reduction

The Methylene Blue Dye Reduction Test is widely used in dairy industry to determine the microbial load in the milk. This test involves the addition of methylene blue into a milk sample and measuring the time required for decolourisation. The disappearance of the colour indicates a high microbial load [17]. Methylene Blue act as a redox indicator that loses its colour under the absence of oxygen. Microbial load causes low oxygen concentration and fast decolourization of methylene blue. The decolourisation of samples in the presence of methylene blue exhibited in fig-2. The sample without hydrogen peroxide changed colour within 6 hours of keeping room temperature whereas sample with 0.2% hydrogen peroxide exhibited delayed decolourization. The sample E decolourised keeping 32hrs in room temperature. MBR test clearly verified the inhibiting properties of H_2O_2 towards the bacterial growth in treated milk and slow microbial load in milk samples containing hydrogen peroxide.

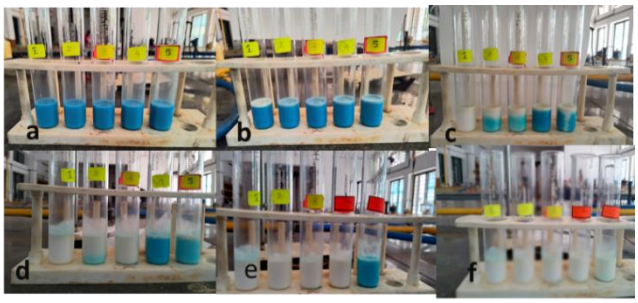


Image 2: Methylene Blue Dye Reduction Test

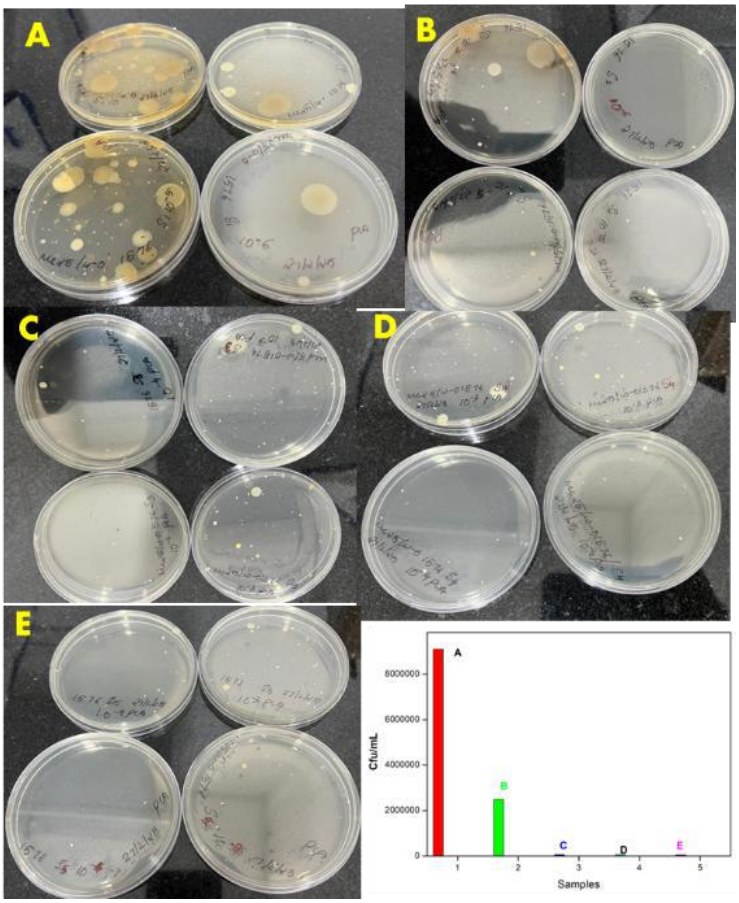


Image 3: Antimicrobial analysis

3.4 Anti-Microbial Analysis

Anti-microbial analysis of milk samples was carried out using Colony forming Unit (CFU) Methode. The result showed in Fig3. As shown in figure, sample without hydrogen hydrogen peroxide (A) exhibited maximum microbial growth. This result is matching with all other observations. When the amount of hydrogen peroxide increased bacterial growth gradually decreased. The enhanced antimicrobial activity of milk samples is attributed to improved activity of lactoperoxidase system in the milk. Lactoperoxidase is a natural enzyme present in the milk, which has inherent ability to protect milk from pathogens. The raw milk which containing greater amount

of lactoperoxidase will have longer shelf life. The amount of lactoperoxidase vary with a species, cow milk conatin an average of 1.4UAmL^{-1} lactoperoxidase. The antimicrobial activity is the combined effect of LP, SCN^{-1} produced by hepatic metabolism and hydrogen peroxide.the SCN^{-1} ion is oxidised by H_2O_2, the reaction is catalysed by LP. The two oxidation products hypothiocyanic acid (HOSCN) and hypothiocyanite ion (OSCN^{-1}) inhibits the growth of microorganisms in the milk. These compounds destroy or modify microbial cell walls leading to death or inhibition of growth of microorganisms[18-20]. The lactoperoxidase system has broad spectrum antimicrobial activity, kill or inhibit bacteria, fungi and virusus. Mamelian cells are not affected by oxidation products of SCN^{-1} and it is suggested that the addition of H_2O_2 is safe to some extent. This fact has greater relevance in places which have no facilities of quick cooling to preserve raw milk.

3.5 Detection of H2O2:

A one step spot detection method for H_2O_2 in raw milk was developed. Four drops hydrogen peroxide testing mixture added to all samples of milk. The sample without hydrogen peroxide didn't show any significant colour change when testing mixture was added dropwise. All other samples containing hydrogen peroxide exhibited colour changes and foams. More the hydrogen peroxides more the quantity of foams generated. All experiments were repeated three times. The KI in the testing mixtures was oxidised to I_2 in the presence of H_2O_2 present in the milk. Meantime H_2O_2 get reduced and oxygen gas released. This oxygen gas leads to foams in the presence lather forming content of testing mixture. The yellow colour is attributed to the presence of I_2. The method is simple and can be practiced anyone. When the amount of hydrogen peroxides increased intensity of yellow colour and hight of foam will also increase.

IV. CONCLUSION

There are situations when milk cannot be preserved in refrigerator, Hydrogen peroxide can act as effective chemical preservative. Raw milk naturally contains low concentration of H_2O_2, which has active role in the shelf life of the raw milk. The addition of optimum amount H_2O_2 in to raw milk has excellent preservative power boosting the activity of LP system naturally present in the raw milk. In the present study, we prepared four different samples with different concentrations of hydrogen peroxide. It was observed that, at the room temperature the increased level of H_2O_2 leads to delay in sour flavour development, positive COB test, decolorization of methylene blue and acidity development. The bacterial count study clearly proved the inhibition effect of hydrogen peroxide in treated sample. The study developed simple qualitative method for the determination of hydrogen peroxide in the raw milk**.**

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