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INVESTIGATING THE EFFECT OF LACTOPEROXIDASE SYSTEM ON KEEPING QUALITY OF RAW GOAT MILK: CASE STUDY ON GOAT MILK FROM BISHOFTU, ETHIOPIA

A THESIS SUBMITTED TO THE JIMMA UNIVERSITY, JIMMA INSTITUTE OF TECHNOLOGY, IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF DEGREE OF MASTER OF SCINCE IN CHEMICAL ENGINEERING (PROCESS ENGINEERING)

By: Abadr Adem

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This is to certify that the thesis prepared by **Abadr Adem**, entitled: *"**Investigating the Effect of Lactoperoxidase system on Keeping Quality of Raw Goat milk: Case study on Goat milk from Bishoftu, Ethiopia**"* and submitted in partial fulfillment of the requirement for the degree of Master of Science (Chemical Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Dr. Kumsa Delessa

_____ _____ _____

External Examiner Signature Date

Mr. Mohammed Seid

_____ _____ _____

Internal Examiner Signature Date

Mr. Abrha Gebremeskel

_____ _____ _____

School or Center of Chairperson Signature Date

Mr. Samuel Gesesse

_____ _____ _____

Program Coordinator Signature Date

Mr. Ermias Girma

_____ _____ _____

Dean, School of Chemical Engineering Signature Date

Declaration

I declare that this thesis entitled: "Investigating the Effect of Lactoperoxidase system on Keeping Quality of Raw Goat milk: Case study on Goat milk from Bishoftu, Ethiopia" has not been submitted in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and list of references is given. The work was under the guidance of Dr. K. Ramachandran, Assistant Professor in School of Chemical Engineering in (JIT), Jimma University and Mr. Yasin Ahmed Lecturer in School of Chemical Engineering (JIT), Jimma University.

Name: Abadr Adem

Signature: _____

Date: _____

This thesis has been submitted for examination with my approval as University Advisor and Co-advisor.

Name: Dr. Ramachandran Kasirajan

Signature: _____

Date: _____

Name: Mr. Yasin Ahmed

Signature: _____

Date: _____

Abstract

Milk is an important source of nutrients for humans and animals. But it is a perishable food that can be spoiled easily and also becomes an important vehicle for transmission of pathogenic microorganisms to human beings. The main goal of this study was to preserve raw goat milk by keeping its quality using the lactoperoxidase system without using the refrigeration system which in effect to deliver safe milk to consumers and to maximize the income by minimizing loss of milk due to spoilage. The composition analysis of raw goat milk and the effects of process parameters on the activation of lactoperoxidase system were also investigated. The lactoperoxidase system was activated by increasing the concentration of sodium thiocyanate and hydrogen peroxide reacting with each other to yield antibacterial compound. The experiment was designed by design expert software 11.00 using central composite design to evaluate the effect of process variables on the growth of total coliform count. The minimum growth of total coliform count 2.658 log CFU/ml were found at the optimal condition of 25 ^{o}C temperature, 4hr time, 29.98 PPM of sodium thiocyanate and 16.6 PPM of hydrogen peroxide. The process variables, had a significant ($\boldsymbol{p} < 0.05$) effects on the growth of total coliform count. The physiochemical characterization (protein content, fat content, lactose content, total solids, moisture content and ash content) of the preserved milk under optimal state were found to be 3.84%, 5.61%, 4.68%, 14.98%, 85.02% and 0.85% respectively.

Microbiological analysis and physicochemical characterization of the raw and preserved goat milk was analyzed using Minitab 17 version. Therefore the lactoperoxidase system does not induce any significant adverse effects on the physicochemical characteristics of raw goat milk. Activation of the lactoperoxidase system can keep goats' milk fresh for up to 10 hr when stored at 25^{0}C.

***Keywords**: Goat milk, lactoperoxidase system, total coliform count, Central composite design (CCD), physicochemical characterization*

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List of Abbreviations

AAiT	Addis Ababa Institute of Technology
AOAC	Association of official analytical chemists
ANOVA	Analysis of variance
APHA	America public health association
CAC	Codex alimentarius commission
CFU	Colony-forming unit
CSA	Central statistical authority
CCD	Central composite design
COB	Clot on boiling
DF	Degree of freedom
DNA	Deoxyribonucleic acid
EEC	European economic community
ES	Ethiopian standards
FAO	Food and agriculture organization
H_2O_2	Hydrogen peroxide
HOSCN	Hypocyanous acid
ILCA	International livestock center for Africa
ISO	International organization for standardization
JIT	Jimma institute of technology
LP	Lactoperoxidase
LPS	Lactoperoxidase system
N	Normality

OSCN	Hypothiocyanate ion
PPM	Parts per million
PW	Peptone water
RNA	Ribonucleic acid
RSM	Response surface methodology
SAIB	South African indigenous breed
SCN	Thiocyanate
$(SCN)_2$	Thiocyanogen
SNF	Solids not fat
SPC	Standard plate count
TBC	Total bacterial count
TCC	Total coliform count
TS	Total solids
VIF	Variance inflation factor
WHO	World health organization

1. Introduction

1.1 Background

Milk is an important source of nutrients for humans and animals and it is meant to be the first and the only food for the offspring of mammals as an almost complete food. It contains in a balanced form of all the essential and digestible components for building and maintaining the animal and human body (Connor 1995).

Goat's milk is one of the types of milk, which is the most complete food known which is highly compatible and nourishing natural food. So it is highly nutritious that it can serve as a substitute for a meal (Getaneh et al., 2016). The main composition of goat milk is water (87 – 88%); the remaining part is total milk solids (Connor 1995). The average gross composition of goat milk is given in Table 1.1.

Table 1.1 The average gross composition values of goat milk

Component	Quantity (%)	Total solids %	Solids not fat %
water	87.7		
Fat	4.5	12.3	
Protein	2.9		7.8
Lactose	4.1		
ash	0.8		

(Zenebe et al., 2014)

This composition is not constant; the average percentage composition of milk components varies with species, breeds of animal, season, feeds, stage of lactation , health and physiological status of a particular animal (Lai et al., 2016; Park 2006; Raynal-ljutovac et al., 2008).

Milk in developing countries (such as Ethiopia, Kenya and Sudan) is transported to collection centers (Lambert 2001). From the collection centers, it is then sent to processing plants by unrefrigerated trucks. There is a delay between milking and processing which can exceed six hours (Lambert 2001); The lack of modern storage facilities and refrigerated transport can contribute to difficulties in preserving milk in developing countries (Nigussie and Seifu 2008). In countries with high ambient temperatures bacterial growth is accelerated at these

temperatures, affecting the bacteriological quality of milk, causing deterioration and affecting the shelf life and safety. The most commonly used method to stop or retard the deterioration of milk on its way from the farmer to the customer and dairy is cooling (Boulares 2011). However, in many parts of the world, this is not possible for various reasons, such as lack of available capital, lack of electricity, less developed road systems, high operational costs, frequent breakdowns of equipment, lack of spare parts and difficulties in the repair of equipment in rural areas (Seifu et al., 2005).

In many parts of Ethiopia, milk is preserved by smoking milk vessels using wood splinters of Oleaafricana to impart desirable aroma to the milk. It was also found to lower the microbial load of raw milk. Smoking of milk vessels is the major method that is traditionally used to preserve raw milk, under these situations; the chance of spoilage of milk is very high (Alemu and Girma 2018).

Therefore, Alternative methods are required to address this problem. One of the process that can be applied to delay milk deterioration is the lactoperoxidase system (LPS) (Zapico et al. 1998). It is activated by increasing the concentrations of two components or activators (sodium thiocyanate and hydrogen peroxide) reacting with each other. This reaction is catalyzed by the enzyme lactoperoxidase which is naturally present in milk and leads to the formation of antibacterial compounds (Bafort et al. 2014; WHO. 2005; Zapico et al. 1998).

The lactoperoxidase (LP) system consists of three components: LP, thiocyanate ion, and hydrogen peroxide (H_2O_2) (Dajanta et al., 2008). They are active only in the presence of all these three components. Lactoperoxidase catalyzes the oxidation of thiocyanate by H_2O_2 and generates intermediate products with antibacterial properties. These products have a broad spectrum of antimicrobial effects against bacteria, fungi, and viruses (Naidu 2000; Wolfson and Sumner 1993).

Milk is a perishable material that can be spoiled easily. The most commonly used method to stop or retard the deterioration of milk on its way from the farmer to the customer and dairy is cooling. However, this is difficult due to different reasons, therefore the aim of this thesis is to extend the shelf life of the milk in areas where refrigeration system is not available and characterization of the preserved milk and investigating the effect of temperature, time, the concentration of NaSCN and H_2O_2 on the bacterial growth and quality of milk.

1.2 Statement of the problem

The world still faces big problems related to foodborne diseases associated with contamination of the food supply. The WHO (2000) reports that each year around 1.3 million cases of active diarrhea reported in children less than five years in the developing countries. A large number of these cases are due to microbial contaminated foods, such as milk. Not only diarrhea there are a lot of diseases transmitted via contaminated milk such as tuberculosis, typhoid fever, dysentery, diphtheria, septic sore throat, and other streptococcal infections, while reports on the microbiological quality and safety of milk and milk products from Ethiopia are scanty (Tesfaye et al., 2011). It should also be noted that many foods are lost as a result of spoilage caused, among others, by spoilage organisms. The total volume of milk production in Ethiopia increased from time to time. According to Getaneh et al., (2017), in Ethiopia 50,501 tonnes per year of goat milk would be produced. However, milk is a perishable material that can be spoiled easily, because of that we cannot share the products to customers and dairy processing plants easily, a lot of milks lost and also it becomes an important media for transmission of diseases to human beings, to prevent such kind of problems we have to use lactoperoxidase system to deliver the product to customers and supplying to dairy processing plant by keeping the quality of milk in areas where refrigeration system is not available.

Considering the fact that up to now only limited research has been performed by different researchers, there is no any optimization of the lactoperoxidase system in goat milk. Therefore, this study is conducted to study the effect of LPS on extending the shelf life of raw goat milk.

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1.3 Objectives

1.3.1 General objective

- The general objective of this study is to preserve raw goat milk by keeping its quality using the lactoperoxidase system

1.3.2 Specific objectives

- To characterize the chemical compositions of raw goat milk
- To study the effect of temperature, time, the concentration of sodium thiocyanate and hydrogen peroxide on preserving the bacterial growth and quality of milk
- To determine the microbiological, physicochemical composition and the quality indicators of preserved milk

1.4 Significance of the study

The importance of this study was the contributing method of preservation of raw goat milk using the lactoperoxidase system in areas where the refrigeration system is not available. This study has a great contribution in terms of preserving milk from spoilage, preventing foodborne diseases transmitted through milk, to increase the availability of quality milk marketing with benefits for both milk producers and consumers, and it reduces also the loss of milk due to spoilage. These studies also contributed that the possibility of getting the appropriate operating conditions for the preservation of the goat milk using the lactoperoxidase system. Preservation of the goat milk using LPs, also play a major role to deliver quality goat milk to be transported from the collection center to the customer and dairy processing plants, preventing loss of money due to spoilage and create job opportunity. The result of this study will be contributed as baseline information for future studies.

1.5. Scope of the Study

This research starts from characterisation of raw goat milk and to preserve the raw goat milk with the help of the lactoperoxidase system in areas where the refrigeration system is not available. Even though, the growth of total coliform count was optimized with respect to storage temperature, storage time, sodium thiocyanate and hydrogen peroxide concentration. Later this preserved goat milk was analyzed its microbiological analysis, physicochemical properties and the quality indicators of preserved milk. Some of the basic properties like total bacterial count, total coliform count, protein content, fat content, lactose content, acid content, total solids, moisture content, solids not fat , ash content, alcohol test, pH and clot on boiling test are determined to characterize the quality of the preserved goat milk. Finally, the obtained result was discussed and the conclusion was drawn based on the result. If there were any necessary things that need additional study and investigation related to this topic, but cannot be achieved in this research paper, were recommending those to future researchers.

2. LITERATURE REVIEW

2.1 Introduction

Milk is the fluid secreted by mammals for the nourishment of their offspring (Kannaiyan et al., 2018) Since humans began to domesticate lactating animals, milk and milk products have been part of the human diet (Geigl 2008). It is considered one of the most complete sources of nutrients for human beings because of its diverse components, such as proteins, vitamins, and minerals that are important in human nutrition (Machinski 2009; Nascimento et al., 2017).

World goat milk production has increased from time to time; it's increasing from 12 million tonnes in 1993 to nearly 19 million tonnes in 2017 (FAO 2019). In 2017, Asia produced 57%, Africa 24%, and Europe 15% of the global goat milk supply. Even though in Ethiopia 50,501 tons of goat milk per year would be produced (Getaneh et al., 2017). However, in developing countries about 20 % of milk produced is lost because of poor microbial quality (Lambert 2001) because of this; techniques that can decrease the microbial load of milk and extend shelf life are required.

In Ethiopia alone, there were 21,961,000 goats. While the number of goats used for milk is highest in the Oromia Region, the Afar Region has the highest proportion (20.92 percent) of the total national goat population (CSA 2010a).

Table 2.1: Number of goat and goat milk

Region	Total goats, ‘000	Milking goats, ‘000	% Milking goats, ‘000
Tigray	2 621	5	0.19
Afar	961	201	20.92
Amhara	4 878	6	0.12
Oromia	7 346	319	4.34
Somali	1 509	73	4.84
Benishangul-Gumuz	336	-	-
SNNP	4 057	52	1.28
Gambela	37.8	0.7	1.85
Harari	41.3	-	-
Dire Dawa	172.9	3	1.74
Ethiopia	21961	660	4.41

(CSA 2010a)

For people with cow milk allergies, cow lactalbumin, and gastrointestinal disorders, goat milk is an ideal substitution (Haenlein 2004). Its health benefits are having low levels of cholesterol and have higher levels of essential amino acids, which if consumed, meet or even exceed the daily dietary requirements (Haenlein 2004). It differs from cow milk in having better digestibility (Park 2000), higher alkalinity, higher buffer capacity, and its particular therapeutic value in medicine and human nutrition (Aganga et al., 2002; Aparnathi and Mehta 2017; Haenlein 2004).

2.2 Microbiological hazards associated with raw goat milk

Fresh goat milk drawn from a healthy ruminant is not sterile but has a low microbial load (Varnam and Sutherland 1994). Contamination of freshly drawn milk may come from the environment, equipment, dust, personnel, transport or the water source (Connor 1995). Goats, they may have much safer milk with lower microbial counts than cow milk. Bacteria in freshly drawn milk may also increase due to mastitis as bacteria may enter through the teat duct and maybe shed into the milk (Verraes et al., 2015). Generally, in fresh goat milk, microbial load is less. However, the count might increase when stored at ambient temperature for an extended period of time (Migeemanathan et al.,2011; Yee et al., 2004).

There are some pathogenic and spoilage bacteria which have been isolated from fresh raw goat milk in different parts of the world such as *Listeria monocytogenes, Escherichia coli, Campylobacter, Salmonella sp, Staphylococcus aureus, Bacillus cereus,* and species of *Streptococcus* and *Micrococcus* (Adesiyun et al., 2007; Eglezos et al., 2008).

Therefore, there are several diseases caused by those microorganisms that are associated with milk and milk products (Table 2.2). Some of the major ones, particularly those associated with milk are briefly discussed below.

***Salmonella*:** Many strains of *salmonella* can cause foodborne illnesses in humans, and all strains exhibit similar symptoms such as gastroenteritis (vomiting and diarrhea)(Jay 2000). When the milk containing salmonella treated with the LP system showing both bacteriostatic and bactericidal activities against salmonella (Wolfson and Sumner 1994).

Listeria monocytogens: *L. monocytogenes* is a Gram-positive, rod-shaped, non-spore forming, and facultative anaerobe bacterium (Cooper 2009). It is widespread in the environment, and control of *Listeria* in food production facilities requires constant focus by managers (Buchanan et al., 2017). *L. monocytogenes* is an important pathogenic bacterium

Table 2.2 Human microbial pathogens associated with milk and milk products

Organism	Disease
Enterobacteriaceae	
Escherichia coli, including O157:H7	Gastroenteritis, hemolytic uremic syndrome
Salmonella	Gastroenteritis, typhoid fever
Yersinia enterocolitica (psychrotrophic)	Gastroenteritis
Other gram-negative bacteria	
Aeromonas hydrophila (psychrotrophic)	Gastroenteritis
Brucella spp.	Brucellosis (Bang's disease)
Campylobacter jejuni	Gastroenteritis
Pseudomonas aeruginosa	Gastroenteritis
Gram-positive spore formers	
Bacillus cereus	Gastroenteritis
Bacillus anthracis	Anthrax
Clostridium perfringens	Gastroenteritis
Clostridium botulinum	Botulism
Gram-positive cocci	
Staphylococcus aureus	Emetic intoxication
Streptococcus agalactiae	Sore throat
Streptococcus pyogenes	Scarlet fever/sore throat
Streptococcus zooepidemicus	Pharyngitis, nephritic sequelae
Miscellaneous gram-positive bacteria	
Corynebacterium spp	Diphtheria
Listeria monocytogenes (psychrotrophic)	Listeriosis
Mycobacterium bovis	Tuberculosis
Mycobacterium tuberculosis	Tuberculosis
Mycobacterium paratuberculosis	Johne's disease (ruminants)
Fungi	
Molds	Mycotoxicoses

(Mart and Steel 2001)

for humans and animals and causes public health problems (Moosavy et al., 2014). As reported by Seifu (2004) the LP system showed a bactericidal effect against *L. monocytogenes* in Saanen and South African indigenous goat milk.

***Staphylococcus aureus*:** is a common cause of mastitis and can enter the milk supply from sores on the teats or from the hands and nasal discharges of dairy farmers and workers. The organism produces an enterotoxin (toxins causing vomiting and diarrhea) in raw milk.

Sufficient amounts of enterotoxin in foods can cause illness (Jay 2000). The LP system showed both bactericidal and bacteriostatic effect against *S. aureus* ATCC 25923 in goat milk (Seifu 2004).

E.coli: *E. coli* is one of the most important pathogenic bacteria, which is a normal resident of large intestine in human and warm-blooded animals (Lara et al., 2016). Therefore, *E. coli* can be transmitted to raw milk and milk products by fecal contamination during the milking process along with poor hygienic practices (Garbaj et al., 2016; Sharafati-chaleshtori et al,. 2014). But the LP system showed a bacteriostatic effect against *E. coli* in Saanen and South African indigenous goat milk (Seifu 2004).

2.3 Quality indicators of milk

Different organizations have been working in different countries in establishing quality standards to ensure the health of consumers. Health hazards to the consumer are often grouped into microbiological, physical and chemical (Fombad 2011).

2.3.1 Microbiological quality of milk

Due to several reasons, it is practically difficult to isolate all pathogens from goat milk samples. Selected microbial tests, that are indicative of the general bacteriological quality, are regularly conducted to evaluate the microbial quality of milk. These tests are the determination of total bacterial count, coliform count and *E.coli* (Kiiyuki 2003). The total bacterial count refers to all viable microorganisms that could grow aerobically and form countable colonies on plate count agar incubated for 48 hr (Jay 2000; Tekilegiorgis 2018). The result gives a quantitative idea about the microbial load present in the sample in order to evaluate guideline values. In legal matters concerning the acceptability of incoming milk, the total bacterial count is one of the most commonly used microbial quality tests for milk and milk products (Tekilegiorgis 2018). It is a measure of hygienic quality of milk. Excessively high counts are indicative of poor hygiene and may lead to early spoilage of milk that makes it unfit for human consumption. Coliforms are a group of bacteria that comprise all aerobic and facultative anaerobic, gram-negative, non-spore-forming rods able to ferment lactose and produce acid and gas at 32– 35^0C (Jay 2000; Pantoja et al., 2011). Coliforms can be found in the aquatic environment, in soil, and on vegetation but they are commonly present in large numbers in the feces of warm-blooded animals. While coliforms themselves do not normally

cause serious illness their presence is used to indicate that other pathogenic organisms of faecal origin may be present (Lund at al., 2000).

According to the European commission's safety limits of milk in community legislation the total bacterial count should not exceed 10^5 CFU/ml and coliform count less than 100 CFU/ml Council directives 92/46 EEC (1992). O'Connor (1994) also reported that high initial counts (more than 10^5 bacteria ml^{-1}) are evidence of poor production hygiene. A count of less than 100 CFU/ml of Coliform count are considered acceptable for milk intended to be pasteurized before consumption (Ruegg 2003).

2.3.2 Alcohol test

The alcohol test, together with the acidity test, is used on fresh milk to indicate whether it will coagulate on processing. Milk that contains more than 0.21% acid, or calcium and magnesium compounds in greater than normal amounts, will coagulate when alcohol is added. This is due to the instability of the proteins when the levels of acid are increased (Connor 1995; Draaiyer et al., 2009).

2.3.3 Clot on Boiling

This is one of the oldest tests for abnormal acidity levels in milk; the clot-on-boiling test is used to determine whether milk is suitable for processing, as it indicates whether milk is likely to coagulate during processing (usually pasteurization). The test is performed by boiling a small amount of milk in a spoon, test tube or any other suitable container. If there is coagulation or precipitation, the milk fails the test. The test is not sensitive to slightly sour milk (Connor 1995; Draaiyer et al., 2009).

2.3.4 Milk composition quality test

Compositional characteristics are the features of raw milk-related to the natural composition that has special importance in processing e.g. fat, proteins, lactose, total solids and solids-not-fat (SNF). Determination of acid in milk is also an important factor in judging milk quality because acidity percentage is a measure of freshness and bacterial activity in milk (Belitz and Le 2009; Saha and Ara 2012).

2.4 Lactoperoxidase system

Milk is a perishable food that can be spoiled and deteriorate easily. The most commonly used method to stop or retard the deterioration of milk on its way from the farmer to the dairy is

cooling (Seifu et al., 2003). However, it is difficult to use due to technical and economic reasons. Therefore an alternative method which is the Lactoperoxidase system has been used to preserve raw milk quality because these techniques are required to be simple to use, relatively inexpensive and not represent any risk to consumers (Srisaikham et al., 2017). preservation of raw milk, pasteurized milk, cheese and yogurt using LPs is familiar in the dairy industry (Lewis 2005; Touch et al., 2004).

The LPs are made up of three components: the enzyme LP, thiocyanate ion (SCN^-) and hydrogen peroxide (H_2O_2) (Ndambi et al., 2008). It can be activated by means of increasing the concentrations of two components (hydrogen peroxide and thiocyanate), reacting with each other (Boulares 2011). This reaction is catalyzed by the enzyme lactoperoxidase which is naturally present in milk and leads to the formation of antimicrobial compounds (Naidu 2000; Wolfson and Sumner 1993). Preservation of the milk using low levels of thiocyanate and hydrogen peroxide, LP exhibits very strong bactericidal activity (Fox and Kelly 2006).

So in warm climates, whereas the refrigeration equipment is not easily available LPs can be applied or in combination with conventional preservation treatments to inhibit pathogenic microorganisms (WHO 2005). The Lactoperoxidase system is permitted to use as preservation for milk and milk products by WHO (2005) and even also in Ethiopia According, to ES 3478 (2009) it is allowed to use the LP system to preserve raw milk.

Therefore in this thesis, lactoperoxidase system method was selected to extend the quality of goat milk because these methods are simple to use, inexpensive and do not have any significant effect on the milk and consumers.

2.5 Components of the lactoperoxidase system

2.5.1 Lactoperoxidase

The lactoperoxidase is an enzyme naturally present in milk that catalyzes the oxidation of thiocyanate ion by hydrogen peroxide to hypocyanous acid (HOSCN) and hypothiocyanate ion (Kussendrager and Hooijdonk 2000). LP is normally found in a sufficient amount in milk; however, thiocyanate and hydrogen peroxide are the limiting factors and need to be added from exogenous sources to activate the LPs (Seifu et al., 2004). Only the presence of lactoperoxidase enzyme has no antimicrobial activity effect, but in combination with H_2O_2 and thiocyanate ion, it forms a natural antimicrobial system (Banks et al., 1989; Boulares 2011; Esho et al., 2013). The concentrations of LP and thiocyanate in different types of milk are different due to vary with breed, species, udder health and type of feed (Kussendrager and

Hooijdonk 2000). The levels of LP and thiocyanate in different types of milk are given in Table 2.3.

Table 2.3 LP activity and NaSCN concentration in different types of milk

Type of milk	Lactoperoxidase Units/ ml	References	Thiocyanate ppm	References
Cow	1.4	Stephens et al. (1979)	3.2 – 4.6	Bjo¨rck et al. (1979)
Ewe	0.14 – 2.38	Medina et al. (1989)	10.3 - 20.6	Medina et al. (1989)
Goat	1.55	Zapico et al. (1990)	4.03	Zapico et al. (1990)
	4.45	Saad de Schoos et al. (1999)	10.29	Saad de Schoos et al.(1999)
SAIB	0.26	Seifu et al. (2004b)	4.58	Seifu et al. (2004b)
Saanen	0.79	Seifu et al. (2004b)	2.78	Seifu et al. (2004b)
Buffalo	0.9	Harnulv and Kandasamy (1982)	5.4	Harnulv and Kandasamy (1982)
Guinea pig	22	Stephenes et al. (1979)	-	
Human	0.06 – 0.97	Reiter (1985)	2.6	Metwally and Nasr(1992)
	0.02	Reiter and Harnulv (1984)	15	Bjo¨rck et al. (1979)

(Seifu et al., 2005)

2.5.2 Thiocyanate (SCN)

Thiocyanate is one of the components of the LPs which is widely distributed in animal tissues and secretions (Wolfson and Sumner 1993). It is present in the mammary, salivary and thyroid glands and their secretions, in organs such as the stomach and kidney (Reiter and Harnulv 1984). It also occurs naturally at low concentrations in milk which are not adequate for inhibition (Bjorck et al. 1998). The minimum thiocyanate amount required to activate the lactoperoxidase system in milk is 15 PPM (Chakraborty and Chaudhry 1985). However, the thiocyanate content is found in a limiting factor and needs to be added from an exogenous source (such as sodium thiocyanate) to activate the LPs (Seifu et al., 2005).

2.5.3 Hydrogen Peroxide

Hydrogen peroxide is the third component of the LP system, which is not normally detected in raw milk (Boulares 2011). Activation of the LP system by the addition of hydrogen peroxide or other hydrogen peroxide generating systems like sodium percarbonate the bacterial spectrum is enhanced (WHO 2005).

Hydrogen peroxide is highly toxic to mammalian cells. However, at low concentrations and in the presence of lactoperoxidase enzyme and thiocyanate concentration mammalian cells are protected from this toxicity (Pruitt & Kamau 1991).

2.6 Reaction mechanisms of the lactoperoxidase system

The antimicrobial activity of the milk can be activated by the reactions of the three components of the lactoperoxidase system (Boulares 2011). The lactoperoxidase catalyzes the oxidation of thiocyanate ion (SCN^-) by hydrogen peroxide to produce the antimicrobial products (Kussendrager and Hooijdonk 2000). The major intermediate oxidation product of the LP catalyzed oxidation of thiocyanate (SCN^-) is hypothiocyanate ion ($OSCN^-$) which shows the antibacterial activity (Wolfson and Sumner 1993). However, the reaction system is complex and hypothiocyanate ion ($OSCN^-$) may not be directly obtained from the oxidation thiocyanate ion by the lactoperoxidase enzyme catalase (Seifu et al., 2005). But indirectly through the oxidation reaction of other short-lived intermediates which is thiocyanogen $(SCN)_2$, which hydrolyzes rapidly to yield hypothiocyanous acid (HOSCN) (Pruitt & Kamau, 1991). The oxidation of thiocyanate ion by lactoperoxidase is expressed below (Thomas and Aune 1978).

Thiocyanate can be directly oxidized to hypothiocyanate

$$SCN^- + H_2O_2 \xrightarrow{lactoperoxidase} OSCN^- + H_2O \quad \ldots\ldots (2.1)$$

Or indirectly

$$2SCN^- + H_2O_2 + 2H^+ \xrightarrow{lactoperoxidase} (SCN)_2 + 2H_2O \quad \ldots\ldots (2.2)$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + SCN^- + H^+ \quad \ldots\ldots (2.3)$$

$$HOSCN \rightleftarrows OSCN^- + H^+ \quad \ldots\ldots (2.4)$$

The products hypothiocyanous acid (HOSCN) and hypothiocyanate ion ($OSCN^{-}$) are highly reactive oxidizing agents they react and oxidizes rapidly the protein sulfhydryl groups to yield Sulphenyl thiocyanate (R–S–SCN)(Seifu et al., 2005).

$$protein - SH + (SCN)_2 \rightarrow protein - S - SCN + SCN^- + H^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots (2.5)$$

$$protein - SH + OSCN^- \rightarrow protein - S - SCN + OH^- \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.6)$$

The structural damage of microbial cytoplasmic membranes by the oxidation of protein sulphydryl (SH) groups is considered to be the key to the antimicrobial action of the LP system (Lewis 2005). This results in leakage of potassium ions, amino acids, and polypeptides into the medium. Subsequently, uptake of glucose, amino acids, purines, pyrimidine's in the cell and the synthesis of proteins, DNA and RNA are also inhibited (Reiter and Harnulv 1984).

2.7 Effect of LPS on nutritional composition of milk

The LPs allows adequate time for the milk to be transported from the collection point to a processing center without refrigeration. The LP system does not induce any significant adverse effects on the physicochemical characteristics of raw milk and processed dairy products (WHO 2005). The chemical composition of the raw cow milk activated with LP system and the control values protein, fat, total solids, and density had no significant difference in all milk samples(Musa et al., 2013.; Srisaikham et al., 2017).

The major factors that affect the LP system are storage temperature, time, thiocyanate and hydrogen peroxide concentration (WHO 2005; Srisaikham et al., 2017). The efficacy of the LPs continues for a limited period of time, which decreases as the ambient temperature increases (WHO 2005). Further, the antibacterial period is proportional to the SCN^- and H_2O_2 levels and is inversely related to the temperature of the storage of milk (Kumar 1986). The minimum level of 15 ppm of SCN and 10 ppm of H_2O_2 (in equimolar concentration) is required to activate the LP-system in milk (Kumar 1986). Different researchers have studied on extending the quality of the cow milk using LPS. Chakraborty and Chaudhry (1985) used multiple concentration doses of SCN^- and H_2O_2 (15:10 PPM to 105:70 PPM). They depicted that a dose of 75:50 of SCN^- and H_2O_2 *gave* the longest Shelf life of about 15 hrs. On the other hand, Gupta (1985) reported that for the preservation of cow milk up to 16 hrs, the requirement of SCN^-: H_2O_2 was found to be 70:30 ppm.

Even also for preserving cow milks at 4^0c multiple concentration of SCN^-: H_2O_2 was used the adequate concentration thiocyanate ion and hydrogen peroxide concentration (28:60) PPM (Boulares 2011), and (20:40) PPM (Musa et al., 2013) was reported.

But in goat milk considering the fact that up to now only limited research has been performed by different researchers. Such as Gürsel et al., (1999) and Haddadin et al.,(1996) extends the quality of goat milk using the LP system with constant ambient temperature and concentration.

But in this research, all the parametric factors were selected to investigate the effect and optimize the best parameter condition on extending the quality of goat milk using the LP system.

2.8 The antimicrobial activity of the Lactoperoxidase system

The LPs can inhibit the growth and metabolism of different species of microorganisms (Seifu et al., 2005). It is capable of inhibiting viruses, gram-positive bacteria, gram-negative bacteria, fungi, and parasites. The cytoplasmic membrane or the cytoplasm are the major targets of the LPs antimicrobial products (Naidu 2000).

3. METHODOLOGY

3.1 Materials

The sample, raw goat milk, was collected from the Ilica area of Bishoftu town located in the Oromiya region, which is 45 km far away from Addis Ababa having 14^{o}C and 26^{o}C minimum and maximum temperature range, with a mean relative humidity of 61.3%.

The raw goat milk collected from Bishoftu town was kept in icebox containing icepacks and transported to the dairy laboratory of the Ethiopian meat and dairy industry development institute found in Bishoftu in which microbiological analysis were carried out and its physicochemical analysis carried out in Jije laboratory found in Addis Ababa.

Chemicals and reagents

The chemicals that were used in this work were include: sodium thiocyanate (98.5%), hydrogen peroxide(30%), distilled water, ethanol(95%), peptone water (HiMedia laboratories ,India), standard plate agar(HiMedia laboratories ,India), violate red bile agar(HiMedia laboratories ,India), sodium hydroxide (NaOH), phenolphthalein, sulphuric acid, ethyl ether, petroleum ether, ammonium hydroxide, copper sulfate ,potassium sulfate, ammonium sulfate, boric acid, Phosphate buffer.

Equipment

The equipment used during this thesis work were: icebox, incubator, ekomilk, the standard plate counter, safety cabinet, gloves ,fridge, beaker, spirit lamp , pHscan 30, erlenmeyer flask, analytic balance ,measuring cylinder ,petri dish, dry oven, furnace, autoclave, desiccator, burette, pipette, micropipette, conical flasks, cylinder ,water bath, test tubes, mixer, kjeldahl, cuvets and distillation.

Before starting the experiment, the sample was tested for freshness using alcohol test, clot on boiling test, freezing point test, pH and titratable acidity test.

3.2 Checking freshness of the milk sample

3.2.1 Alcohol test

Milk freshness was tested by using an alcohol test. 68% alcohol solution was prepared from 95% alcohol. Then it was mixed with distilled water in the proportion of 71.6 ml of 95% alcohol to 28.4 ml of distil water. Then equal volumes of milk and 68% alcohol put in a test

tube (Dajanta 2008; Draaiyer 2009; Esho et al., 2013; Musa et al., 2013). The principle of the test was milk coagulation which indicates milk quality deterioration and it was considered to be a breakpoint for the shelf life of milk. Milk containing more than 0.21% acid and milk that is abnormal (e.g. colostrum or mastitis milk) would not pass the test. This milk is not fit for further processing.

3.2.2 Clot-on-boiling test

5 ml of milk was placed in a test tube (the exact amount was not important) and the test tube held with a test tube holder. Then the milk sample in the test tube was boiled using a spirit lamp for 2–3 minutes. The test tube was carefully removed and examined for precipitate. The milk was rejected if any curd forms (Connor 1995).

3.2.3 Titratable acidity test

The percentage of acid present in milk is a rough indication of its age and the manner in which it has been handled.it determines the suitability for processing. The burette was filled with 0.1 N of NaOH and then the level of NaOH was adjusted in the burette to the top mark, the lowest reading was at the upper end, 10 ml of milk was delivered into the beaker and 3 to 5 drops of phenolphthalein was added into the milk sample. NaOH was allowed to flow slowly into the beaker containing the sample and mixing continuously. When a faint but definite pink color persists, the end-point had been reached. Then the reading of the burette was taking at the lowest point of the meniscus. the number of milliliters' of alkali (NaOH) required to neutralize the acid in the sample was determined by subtracting the first reading from the second (Connor 1995).

$$Lactic\ acid(\%) = \frac{ml\ alkali \times 0.009 \times 100}{ml\ of\ sample} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \ (3.1)$$

3.2.4 pH value

pH value is one of the quality indicators of raw goat milk. This could be determined by PHscan 30 equipment. The equipment PHscan 30 first calibrated by a buffer solution having a pH 4.3 @ 25^{0}C then it was cleaned and rinsed by distilling water. Then it was placed on the raw goat milk sample to determine the pH of the milk sample and the result that displayed on the screen was recorded

3.2.5 Freezing point test

The freezing point test is a test for the adulteration of milk with water. This could be done using Ekomilk equipment which is more accurate to determine the adulteration of milk with water than the lactometer test.

The ekomilk equipment first calibrated using distilled water, then the raw goat milk sample placed and directly analyzed using ekomilk equipment and the result displayed was recorded.

- All the major components of the raw goat milk (protein content, fat content, total solid, solids not fat content, lactose content and ash content) were determined in order to compare the significant difference with preserved milk.

3.3 Method

3.3.1 Activation of LPS

After checking milk freshness lactoperoxidase was activated by adding (15 - 30) PPM sodium thiocyanate as a source of SCN^- to increase the SCN^- level, After 1 minute of thorough mixing (10 - 20) PPM of hydrogen peroxide was added in 100 ml of milk respectively(Gupta et al., 1986; Kamau 2010). Then the samples were subjected to different storage temperature conditions of (25 – 37) ^{o}C for 4 - 8 hrs. In order to get (10 – 20) PPM of hydrogen peroxide 3.3ml of 30% H_2O_2 was added to 96.7 ml of distill water to get 1% of H_2O_2 concentration from which 0.1 ml – 0.2 ml of 1% H_2O_2 was taken for LP system activation per 100 ml of milk respectively. Whereas to obtain (15 – 30) PPM of sodium thiocyanate, 0.15 – 0.3 ml was taken from 1% sodium thiocyanate solution and then added per 100 ml of milk respectively.

3.3.2 Microbiological analysis of the processed milk

3.3.2.1 Total bacterial count (TBC)

Total microbial count for milk was estimated using the standard plate count (SPC) method. Each milk sample (1 ml) was transferred into 9ml of sterilizing peptone water (PW) by means of a micropipette and properly mixed together to make the first 10^{-1} dilution. Serial dilution up to 1: 10^{-8} was prepared by progressive transferring of 1ml of the lower dilution into 9 ml of peptone water to yield the next higher dilution. Each dilution was mixed thoroughly by a vortex mixer before 1ml withdrawn for serial dilution and a separate sterile pipette was used

for making each transfer. 12-15 ml of molten sterile Standard Plate Agar (SPC) was added on a Petri dish and 1 ml of the diluted solution was poured and mixed thoroughly. The plated sample was solidified in a safety cabinet and it was put in the incubator at 32^oC in the inverted position for 48 hrs. After which the number of bacterial colonies grown was counted. When the number of colonies was found too many, compromising the accuracy of counting, the same procedure was repeated using higher dilution levels (APHA 1992; Srisaikham et al., 2017).

The following formula is used to calculate the counts:

$$N = \frac{\sum c}{[(1 \times n_1) + (0.1 \times n_2)]d} \quad \text{...} (3.2)$$

Where

N: Number of colonies per ml or g of product

ΣC: Sum of all colonies on all plates counted

n_1: Number of plates in the first dilution counted

n_2: Number of plates in the second dilution counted

d: Dilution from which the first counts were obtained

3.3.2.2 Total coliform Count (TCC)

Each milk sample (1 ml) was transferred into sterilize tubes containing 9 ml of sterilize peptone water in the same way as the above. Serial dilutions of 1/100 and 1/1000 were prepared by progressive transferring of 1ml of the lower dilution into 9 ml of peptone water to yield the next higher dilution. Dilution levels of 1/100 and 1/1000 were selected for culture. Each dilution was cultured in duplicates. After agitation of each dilution sample, 1 ml of the diluted solution was poured on a Petri dish on which 12-15 ml of molten sterile Violet Red Bile Agar (VRB) was added and mixed thoroughly. When the solution in the Petri dish solidified it was put in the incubator at 32^oC in the inverted position for 24 hrs after which the number of bacterial colonies grown was counted. When the number of colonies was found too many, compromising the accuracy of counting, the same procedure was repeated using higher dilution levels (APHA 1992; Marth and Stele, 2001; Srisaikham et al., 2017). The Total Coliforms Count (TCC) was computed from duplicate plates containing between 10 -

250 colonies in the same as described above for computing the Total Bacterial Count. The same formula was also used and the final result was reported as CFU/ml (colony-forming units per milliliter of sample.)

3.3.3 Chemical properties of raw and preserved milk

3.3.3.1 Fat Content

The fat content of the milk samples was determined according to AOAC method by the ether extraction method. Empty fat – extraction flask was weighted, 10 g of the milk sample was Pipetted into fat – extraction flask and weigh again. 1.5 ml NH_4OH was added thoroughly to neutralize any acid present and 3 drops of phenolphthalein indicator was added to help sharpen the visual appearance of the interface between ether and aqueous layers during extraction. Then 10 ml ethyl alcohol was added and shakes the flask for 15 s. For the first extraction, 25 ml ethyl ether and 25 ml of petroleum ether was added to extract the fat with 1 min interval. Then the flasks were centrifuged at 600 rpm for 30 s to obtain clean separation of aqueous (bright pink) and ether phases. Ether solution was decanted into a weighing dish.

For the second extraction, 5 ml ethyl alcohols were added and shake for 15 s, then 15 ml ethyl ether was added and shake for 1 min and also 15 ml petroleum ether was added and shaking for 1 min. Then the flasks were centrifuged at 600 rpm for 30 s to obtain clean separation of aqueous (bright pink) and ether phases. The ether solution were decant for second extraction into the same weighing dish used for the first extraction.

For the third extraction, the addition of ethyl alcohol was omitted and repeating the procedure used for the second extraction. The solvents were evaporated completely in the hood on a hot plate. the extracted fat and weighing dish was dried to constant weight in a forced air oven at 100°C for 30 min. the weighing dishes removed from the oven was placed in desiccator to cool to room temperature. The weight of the dish plus fat was recorded (A.O.A.C Official Method 989.05, 1995).

$$\text{Fat}, \% = \frac{100 \times \{(\text{weight dish} + \text{fat}) - (\text{weigt dish})\}}{\text{weight milk}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3.3)$$

Where the weight measured is in gram

3.3.3.2 Total Solid Content in Milk

The total solid content of the milk samples was determined according to AOAC by forced air oven drying method. 5 g of the milk was transferred into the crucible and weighted. Moisture removal was carried out in a two-stage process. Firstly, it was pre-dried over a steam bath using 38°C for 25 min before drying in an oven and then it was placed inside a drying oven at 100°C for 3hrs. The crucible was placed in desiccators for 15 min after removed from the oven. The weight of the crucible after drying was recorded (A.O.A.C Official Method 925.23, 1995; O'Connor 1995).

$$\text{Total solids \%} = \frac{M_2 - M_0}{M_1 - M_0} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.4)$$

If the total solid content in milk is known the rest is moisture content which can be calculated by the following equation.

$$\text{Moisture content \%} = \frac{[(M_1 - M_0) - (M_2 - M_0)]}{M_1 - M_0} \times 100 \quad \ldots\ldots\ldots\ldots (3.5)$$

Where:

M_0 = the mass, in grams, of the crucible

M_1 = the mass, in grams, of the crucible and sample

M_2 = the mass, in grams, of the crucible and dried sample

3.3.3.3 Solids not fat content

The solids Not fat (SNF %) was determined by subtracting the percent fat from total solids (Mahony 1998).

$$\text{SNF \%} = (\text{TS} - \text{Fat}) \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.6)$$

3.3.3.4 Protein content

The Protein content of the milk sample was determined according to ISO 1871:2013 by the Kjeldahl method in which digestion, distillation, and titration was involved. 10g of potassium sulfate (to increase the boiling point of sulfuric acid) and 0.2g of copper sulfate was added as a catalyst to each digestion tube. 4 ml of 30% hydrogen peroxide as an oxidizing agent was

added slowly. Then 1 g of milk was added to the digestion flask and 25 ml of sulfuric acid was added to each tube, to digest the sample and blank (without sample).

It was digested using 370^{o}C for 3hrs until the solution was clear. The digested was cooled to room temperature and 70 ml of distilled water was added to each tube to cool it. 90 ml of 45% sodium hydroxide solution was added to the cooled digest to liberate ammonia. The flask was immediately connected to the distillation bulb on the condenser. 30 ml H_3BO_3 solution and a few drops of mixed indicator solution were added to a 250-ml conical flask and it was placed under the condenser of the distillation set-up. The sample solution was heated until all NH_3 had been distilled and collected in a boric acid solution. The H_3BO_3 receiving solution was titrated with standard 0.02N hydrochloric acid solution until the color changes from green to violet and the amount of titrant was recorded (ES ISO 1871, 2013).

$$\text{Total Nitrogen \%} = \frac{(V_s - V_b) \times N \times 0.014 \times 100 \times mcf}{SW} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.8)$$

Where:

Vs = ml HCl titrant used for sample N = normality of HCl

Vb = ml HCl titrant used for blank mcf = moisture correction factor

SW = sample weight (g)

% Crude Protein from % Nitrogen content

$$\text{Protein \%} = \text{Nitrogen \%} \times 6.38 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.9)$$

3.3.3.5 Ash content

Ash content of the milk was determined according to AOAC method using a muffle furnace. The crucible was placed in the furnace at 550^{o}C to ensure that impurities on the surface of crucible were burned off. The crucible was cooled in the desiccator and weighed. 5g of the milk sample was poured into the crucible. The sample was heated at 550^{o}C and cool down in desiccator. The ash and crucible were weighed. (A.O.A.C Official Method 945.46, 1995).

$$\text{Ash \%} = \frac{W_2 - W_0}{W_1 - W_0} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.10)$$

Where

W_o - weigh of crucible, in grams

W_1 - weigh of crucible and sample, in grams

W_2 - weigh of crucible and lid plus ash, in grams

3.3.3.6 Lactose content

Lactose content was calculated by subtracting the sum of fat, protein and ash contents from total solids (Azeze et al. 2015).

$$\text{Lactose } \% = \text{Total solids } \% - (\text{Fat} + \text{Protein} + \text{Ash}) \% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.11)$$

3.4 Experimental design

The experiments were designed using Design - Expert software 11.0 to determine the effect of process variables on the growth of total coliform count. The analysis of variance (ANOVA) was performed using Central composite design (CCD) of Design-Expert 11.0 software a mode in RSM (Response Surface Methodology), to investigate the effects of four independent process variables namely, temperature (A), storage time (B),sodium thiocyanate(C) and hydrogen peroxide concentration (D) on the response, total coliform count (Y). A total of 30 experiments were required in accordance with the CCD methodology, including experiments covering the full range of independent variables, of which 24 were factorial points and 6 were on center points. The range of independent variables was coded into low (-1) and high (+1) levels, with the 6 center points repeated to ensure that any kind of experimental error was avoided. In addition, the experiments were performed in a random order so as to decrease the possibility of errors arising from systematic trends in the variables. The software gave 30 runs to support the data in CCD and the following equation was used to calculate the number of experiments (N).

$$N = 2^n + 2 \times n + n_c \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.11)$$

Where

N = number of experiments

n = is the number of independent variables

n_c = is the number of repeated runs.

The interaction between the response variable and independent variables was correlated by an empirical model using a second-degree polynomial equation as shown in Eq. (3.12).

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n=1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \quad \text{.....(3.12)}$$

Where Y is the predicted response, b_0 the offset term, b_i the linear effect, b_{ii} the square effect, and b_{ij} is the interaction effect. It is a polynomial regression model in one variable and is called as second order model or quadratic model.

The analysis of microbiological and physicochemical of the raw and preserved goat milk was performed using Minitab version 17.

Table 3.1 the levels of variables chosen for the trials

Variables	Levels		
	Lower	Medium	Upper
Temperature (^oC)	25	31	37
Time (hr)	4	6	8
NaSCN(PPM)	15	22.5	30
H_2O_2(PPM)	10	15	20

4. RESULTS AND DISCUSSIONS

4.1 Freshness analysis of raw goat milk

The freshness of the raw goat milk and preserved goat milk was analyzed using an alcohol test and COB test. The principle of the test is milk precipitation or coagulation which indicates milk quality. However, the raw goat milk didn't coagulate using the alcohol test and clot on boiling it has a negative response which indicates it is fresh that fits for further processing.

Table 4.1 Freshness analysis of raw and LP activated goat milk

No.	Parameters	Raw goat milk	LP activated milk up to 10 hr
1	Alcohol test	-ve	-ve
2	Clot on boiling test	-ve COB	-ve COB
3	PH	6.6	6.52
4	Freezing point	-0.52	

When the milk was treated by sodium thiocyanate and hydrogen peroxide, the lactoperoxidase enzymes catalyses the oxidation of thiocyanate by hydrogen peroxide to form antimicrobial compounds which kills or retards the growth of bacteria. As a result, the conversion of lactose (development of acids) by bacteria would be decreased and the stability of the protein also maintained. Therefore the results in Table 4.1 shown that preserved (LP activated) goat milk had a negative response they were not coagulated using alcohol test and clot on boiling test up to 10 hr because the acid development by the conversion of lactose is low due to microbial inhibition by the LPs. LP system can keep goats' milk fresh for up to 10 hr but the control milk up to 4 hr without any coagulation or precipitation when stored at 25^0C. According to FAO (2005), the inhibitory effect of the treatment is dependent on the temperature of the stored milk when the temperature was (30, 25, 20, 15)^{0}C keeps the quality of milk up to (7-8, 11-12, 16-17, 24-26) hr respectively carried out in different countries with raw milk of an initial good hygienic standard. This study has slightly confirmed that the preservation of goat milk at 25^oC, 29.98 PPM sodium thiocyanate and 16.6 PPM of hydrogen peroxide can extend the keeping quality of the goat milk up to 10 hr. In contrast to this study

Nigussie & Seifu (2008) and Gürsel et al., (1999) reported that the LPs can keep the quality of goat milk up to 7 hr and 6 hr respectively. The difference of these results is because of so many factors such as difference in initial microbial load of the goat milk, storage temperature, health status of the goat, and the concentration of NaSCN and H_2O_2 used. When higher amount sodium thiocyanate and hydrogen peroxide used the thiocyanate ion and LP activity increases, results in increasing the antimicrobial products that inhibits the microbial (that deteriorates the milk) growth and keeps the quality of the milk for a long time.

Freezing point test

The freezing point test is a test for the adulteration of milk with water. Milk and water have different freezing points; therefore added water in milk can be detected by measuring the freezing point of the sample. Water has a freezing point of 0 °C, whereas milk normally freezes between −0.52 and −0.56 °C due to dissolved components (mainly lactose and salts)(Draaiyer et al., 2009). When it has been adulterated the freezing point rises nearer to that of water. But the raw goat milk has a freezing point -0.52 between the range of -0.52 and -0.56, therefore, the raw goat milk is not adulterated with water.

pH test

pH value is one of the quality indicators of raw goat milk. This was determined by PHscan 30 equipment. The raw goat milk has a pH of 6.73 which is safe for further processing and my finding was slightly higher than 6.70 reported by (Gaddour et al., 2013). Values below pH 6.5 indicate that considerable acid development has taken place. This is normally due to the conversion of lactose by bacterial activity (Connor 1995).

4.2 Effect of lactoperoxidase system on the microbial growth

In this study, the growth of total coliform count on the activated lactoperoxidase system of goat milk was investigated. The results of the total coliform count growth on the activated lactoperoxidase system goat milk are presented in Table 4.2.

Table 4.2 Central composite design matrix and experimental results

Run NO	Factor 1 Temperature (^{o}c)	Factor 2 Time (Hr)	Factor 3 NaSCN (PPM)	Factor 4 H_2O_2 (PPM)	TCC CFU/ml	TCC Log CFU/ml
1	25	8	30	10	7.636×10^2	2.88289
2	37	8	15	20	1.33×10^3	3.12455
3	31	6	22.5	15	7.206×10^2	2.85769
4	25	8	15	20	8.954×10^2	2.95204
5	37	8	30	20	9.202×10^2	2.96387
6	31	6	22.5	15	7.409×10^2	2.86976
7	31	6	22.5	25	8.364×10^2	2.9224
8	37	8	15	10	1.190×10^3	3.07571
9	37	8	30	10	1.05×10^3	3.02119
10	31	6	22.5	15	7.545×10^2	2.87769
11	25	8	15	10	9.249×10^2	2.96613
12	25	4	30	10	5.136×10^2	2.71066
13	37	4	15	10	8.239×10^2	2.91585
14	37	4	30	10	7.545×10^2	2.87769
15	25	4	15	20	4.898×10^2	2.69005
16	31	6	22.5	15	7.364×10^2	2.86709
17	25	4	30	20	4.545×10^2	2.65758
18	31	6	22.5	15	7.768×10^2	2.89029
19	19	6	22.5	15	5.127×10^2	2.7099
20	31	6	22.5	5	9.887×10^2	2.99507
21	25	8	30	20	6.108×10^2	2.78591
22	37	6	22.5	15	8.773×10^2	2.94313
23	31	10	22.5	15	1.121×10^3	3.04978
24	37	4	30	20	7.152×10^2	2.8544
25	37	4	15	20	8.249×10^2	2.91643
26	31	6	22.5	15	7.119×10^2	2.85242
27	31	6	37.5	15	6.273×10^2	2.79746
28	31	2	22.5	15	5.182×10^2	2.71448
29	31	6	7.5	15	8.986×10^2	2.95355
30	25	4	15	10	4.879×10^2	2.68888

The growth of total coliform count for each run of the experiment was determined using Eq 3.2. The varying the growth of total coliform count indicates that the parameters (i.e. temperature, time, NaSCN and H_2O_2) for activating the lactoperoxidase system considerably affect the growth of total coliform count.

From Table 4.2 the growth of total coliform count decreases with increase of sodium thiocyanate and hydrogen peroxide concentration and decreasing temperature and time.

Based on this, the minimum growth of total coliform count was noted at 25^{o}C temperature, for 4 hr, 30 PPM of sodium thiocyanate and 20 PPM of hydrogen peroxide concentration. For this condition, the obtained total coliform count was 2.65758 log CFU/ml. The minimum value obtained for the growth of total coliform count seemed to be lower than 3.48 log CFU/ml reported by (Srisaikham et al., 2017). The maximum growth of total coliform count 3.12455 log CFU/ml was obtained at 37^{o}C temperature, 8 hr, 15 PPM of sodium thiocyanate and 20 PPM of hydrogen peroxide concentration. The maximum value obtained for the growth of total coliform seemed to be lower than 3.851 log CFU/ml reported by (Srisaikham et al., 2017).

The result obtained from this study was slightly different to the reported values and the slightly deviations may come from, difference in initial microbial load of the goat milk, storage temperature, different breed and the method of activation conditions.

The growth of total coliform count increases at high temperature and time and at low concentration of sodium thiocyanate and hydrogen peroxide. This may be due to when the concentration of sodium thiocyanate (NaSCN) and hydrogen peroxide (H_2O_2) decreases the formation thiocyanate ion (SCN^-) and lactoperoxidase activity would be decreased, therefore, the anti-microbial property also decrease (Srisaikham et al. 2017). Even though when the storage temperature and time increase the growth of total coliform count would be increased this was due to coliform bacteria that survive the initial bactericidal activity of the LPs, when they found suitable temperature condition they recover their damaged cytoplasmic membrane and resume their regular growth after recovery. The length of the recovery period is highly temperature-dependent, being much longer at cold storage than at high temperatures (Kamau and Kroger 1984). Therefore, the length of the antibacterial effect achieved by the LPs activation is inversely related to the temperature (FAO 2005).

The decrease and increase of the growth of total coliform count depend on the level of factors. The result data, from Table 4.2, were analyzed using Design expert® 11.0 Software to determine the effect of temperature, time, sodium thiocyanate and hydrogen peroxide concentration. The dependent variable used as a response parameter was the total coliform count. All experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to extraneous factors.

4.3 Determination of statistical model

The statistical analysis for lactoperoxidase activation in goat milk was done using analysis of variance (ANOVA). A multiple regression program was used to derive the relationships between storage temperature, storage time, sodium thiocyanate and hydrogen peroxide concentration, with total coliform count as the response variables. The feasibility of fitting experimental data to a statistical model was checked on a linear model, a two factor interaction model, a quadratic model and a cubic model as shown in Table 4.3.

Table 4.3 Model prediction summary for experimental data

Source	Sequential p-value	Lack of Fit p-value	R^2	Adjusted R^2	Predicted R^2	
Linear	< 0.0001	0.0119	0.9188	0.9058	0.8751	
2FI	0.0292	0.0268	0.9586	0.9368	0.9130	
Quadratic	0.0013	0.1629	0.9866	0.9742	0.9202	suggested
Cubic	0.1216	0.3719	0.9973	0.9871		

The Model prediction (regression model) was found to be highly significant with the correlation coefficients of determination of R^2, adjusted R^2 and predicted R^2 having a value of 0.9866, 0.9742 and 0.9202 respectively in a quadratic model. From Design expert software the model statistics summary was suggested to utilize a quadratic model for the present experimental data as shown in the Table 4.3.

Table 4.4 Analysis of variance (ANOVA) for predicted model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	0.4130	14	0.0295	79.08	< 0.0001	significant
A -Temperature	0.1360	1	0.1360	364.50	< 0.0001	
B - Time	0.1893	1	0.1893	507.35	< 0.0001	
C - NaSCN	0.0328	1	0.0328	88.00	< 0.0001	
D – H_2O_2	0.0048	1	0.0048	12.87	0.0027	
AB	0.0030	1	0.0030	8.02	0.0126	
AC	0.0002	1	0.0002	0.5129	0.4849	
AD	0.0011	1	0.0011	2.91	0.1087	
BC	0.0078	1	0.0078	20.96	0.0004	
BD	0.0001	1	0.0001	0.3382	0.5695	
CD	0.0045	1	0.0045	11.96	0.0035	
A^2	0.0001	1	0.0001	0.3305	0.5739	
B^2	0.0000	1	0.0000	0.0534	0.8204	
C^2	0.0000	1	0.0000	0.0493	0.8272	
D^2	0.0112	1	0.0112	29.92	< 0.0001	
Residual	0.0056	15	0.0004			
Lack of Fit	0.0047	10	0.0005	2.49	0.1629	not significant
Pure Error	0.0009	5	0.0002			
Cor Total	0.4186	29				

Table 4.4 shows analysis of variance (ANOVA) obtained from Design expert software, which tells as the significance of different factors using a quadratic model. The adequacy of the model was further checked with analysis of variance (ANOVA) as shown in Table 4.4 based on 95% confidence level. F-value is a test for comparing model variance with residual (error)

variance. If the variances are close to the same, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response with the P-value less than 0.05. A model p-value of less than 0.05 shows significance of the model at a 95% confidence level, however, lack of fit should be non-significant for appropriate model. Lack of fit is the comparison between the residual error and pure error which should be non-significant for a significant model. However, only a quadratic model was non-significant lack of fit for a significant model. Analysis of variance (ANOVA) for the experimental data with predicted quadratic model is shown in Table 4.4. Due to the Model F-value of 79.08 and p-value of less than 0.05 indicate model terms are significant. The predicted quadratic model was highly significant for this experimental data. There is only a 0.01% chance that an F-value this large could occur due to noise (personal error or disturbance). The Lack of Fit F-value of 2.49 implies the Lack of Fit is not significant relative to the pure error. There is a 16.29% chance that a Lack of Fit F-value this large could occur due to noise (personal error or disturbance). Non-significant Lack of Fit is good we want the model to fit. The Probability Values of "Prob > F" values less than 0.0500 indicate model terms are highly significant. In this case A, B, C, D, AB, BC, CD, and D^2 are significant model terms. However, the interactions AC, AD and BD were insignificant. Values greater than 0.1000 indicate the model terms are not significant responses.

Table 4.5: Model adequacy measures

Std. Dev.	0.0193	R-Squared	0.9866
Mean	2.88	Adjusted R-Squared	0.9742
C.V.	0.6708	Predicted R-Squared	0.9202
		Adeq Precision	34.1278

R-squared (R^2) is a statistic that explains the amount of variance accounted for in the relationship between two (or more) variables. Adjusted R^2 is used to compare the goodness of fit for regression model that contain differing numbers of independent variables. Its value decreases when the term doesn't improve the model fit by a sufficient amount. Predicted R^2 value determines how well a regression model makes prediction. This static helps to identify cases where the model provides a good fit for the existing data or isn't as good at making predictions.

The "Pred R-Squared" of 0.9202 is in reasonable agreement with the "Adj R-Squared" of

0.9742; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 34.1278 indicates an adequate signal. This model can be used to navigate the design space.

4.4 Development of regression model equation

The model equation that correlates the growth of total coliform count to the parameters of the lactoperoxidase system of the goat milk in terms of coded factors was given in Eq. 4.1.

Final equation in terms of coded factors:

$$TCC = + 2.87 + 0.0851\ A + 0.0888\ B - 0.0370\ C - 0.0141\ D - 0.0137\ AB - 0.0035\ AC + 0.0082\ AD - 0.0221\ BC - 0.0028\ BD - 0.0167\ CD - 0.0027\ A^2 + 0.0008\ B^2 + 0.0008\ C^2 + 0.0200\ D^2 \quad (4.1)$$

Where the coded values of the independent variables are;

TCC = total coliform count

A = temperature

B = time

C = sodium thiocyanate

D = hydrogen peroxide

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final equation in terms of actual factors

$$TCC = + 1.90986 + 0.023366\ A + 0.114575\ B + 0.013622\ C - 0.023632\ D - 0.001140\ AB - 0.000077\ AC + 0.000275\ AD - 0.001474\ BC - 0.000281\ BD - 0.000445\ CD - 0.000076\ A^2 + 0.000211\ B^2 - 0.000014\ C^2 + 0.000800\ D^2$$

Where the coded values of the independent variables are;

TCC = total coliform count

A = temperature

B = time

C = sodium thiocyanate

D = hydrogen peroxide

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

4.5 Diagnostics of model adequacy

The adequacy of the model was checked by constructing different diagnostic plots. The plot of actual values of the experiment and predicated values by the model equation is given in Figure 4.1 for virtual comparison of experimental values and predicted values. The values were found in linear arrangement (the points of all actual and predicted responses fell in 45^{o} lines) showing the good agreement of experimental and model results, these indicating good response to the model.

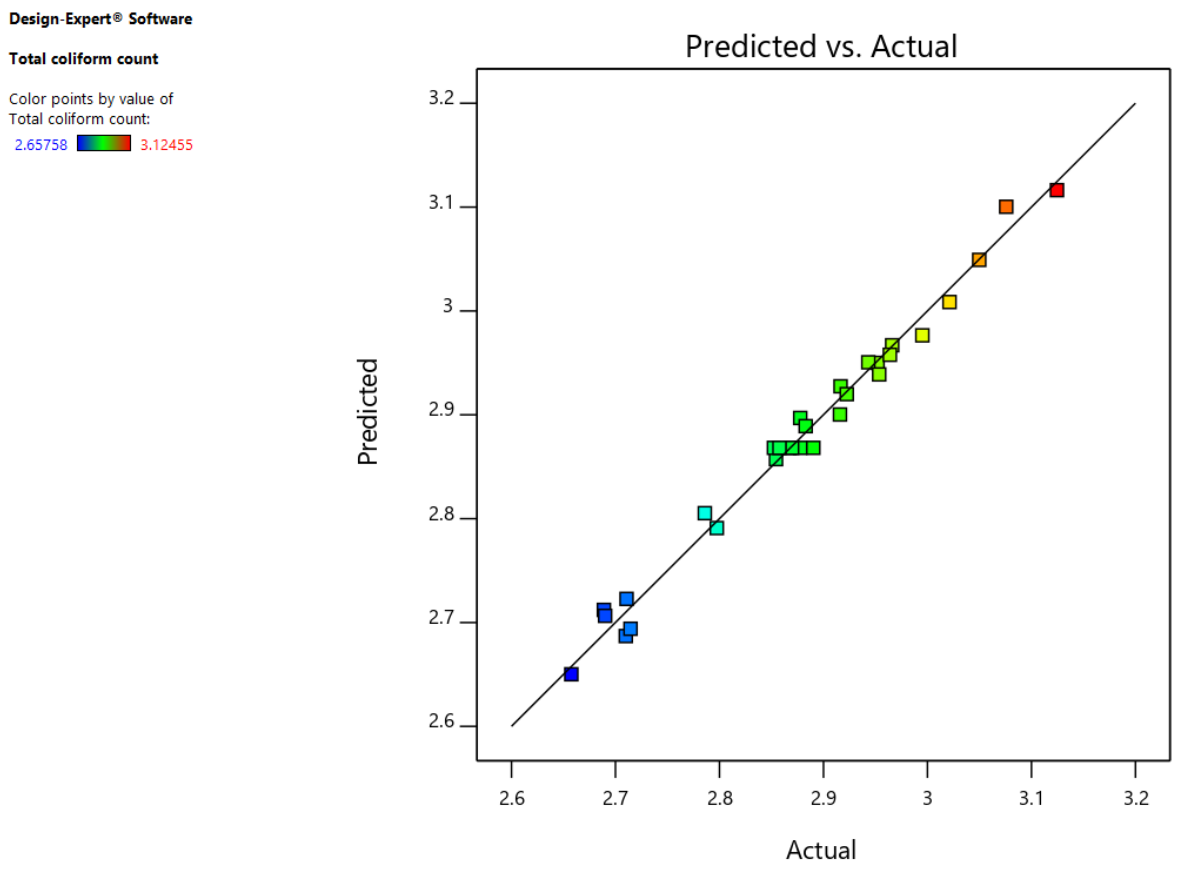

Figure 4.1 Actual value verses predicated value

A residual is the difference between an actual observation and predicted (estimated) values from the statistical model being studied. The normality assumption can be checked by constructing a normal probability plot of the residuals.

A check of the normality assumption could be made by plotting a histogram of the residuals. If assumption on the errors is satisfied, this plot should look like a sample from a normality distribution centered at zero. Unfortunately, with small samples, considerable fluctuation often occurs, so the appearance of a moderate departure from normality does not necessarily imply a serious violation of the assumptions. However gross deviations from normality are potentially serious and require further analysis.

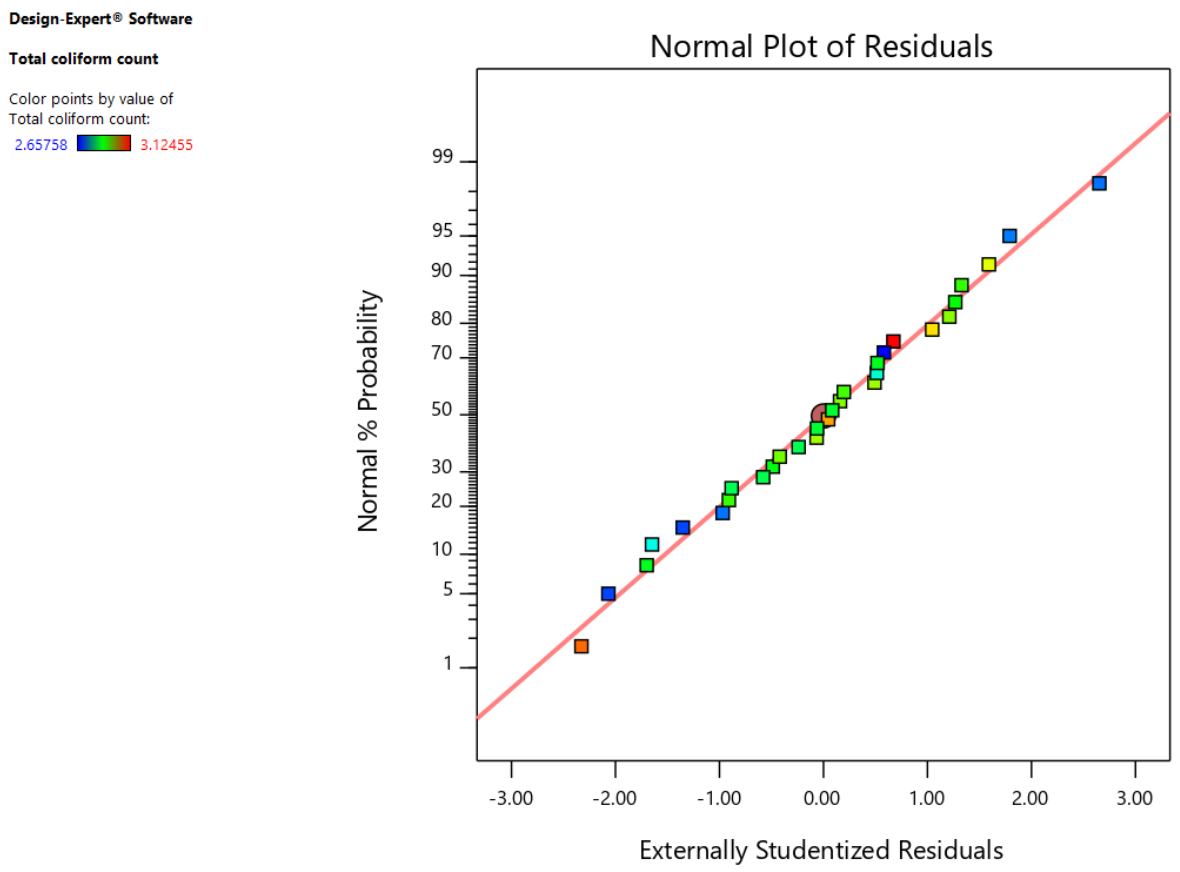

Figure 4.2 Normal plots of residuals

The normal probability plot in Figure 4.2 indicates the residuals following a normal distribution, in the case of this experiment the points in the plots shows fit to a straight line in the figure i.e. the error distribution is approximately normal. This indicates the model satisfies the assumption of ANOVA. If the model is correct and if the assumptions are satisfied, the residuals should be structureless; in particular, they should be unrelated to any other variable including the predicted response. The plot in Figure 4.3 shows that residuals become structureless and this indicates the model satisfies the expected plot of residuals versus predicted response.

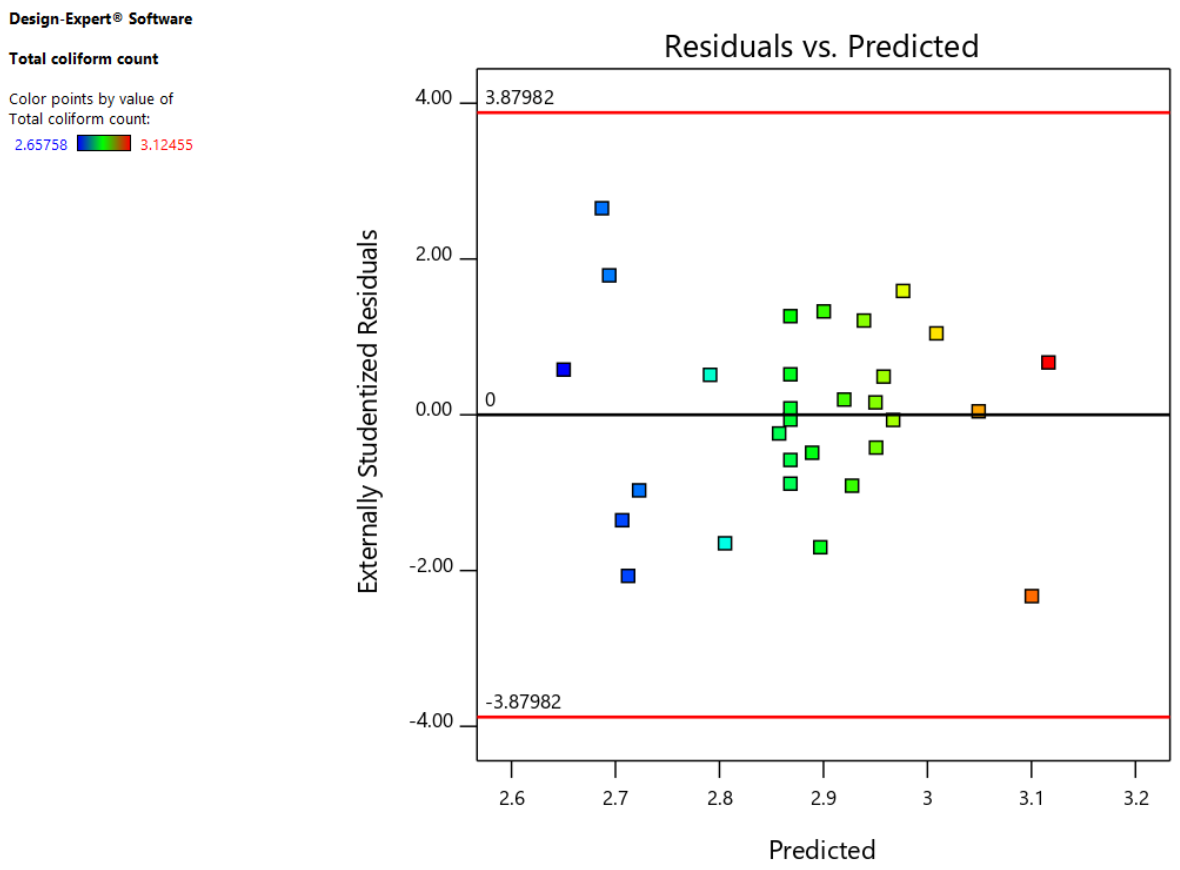

Figure 4.3 Plots of residuals versus predicted

4.6 Individual effect of process parameters on the growth of TCC

The effects of the operating conditions on the coliform growth were investigated and the optimal values were determined in this study.

4.6.1 Effect of temperature on total coliform count

The growth of total coliform count strongly depends on the storage temperature. The effect of temperature on the growth of total coliform count was shown in Figure 4.4. As temperature increases from 25 ^{o}C to 37 ^{0}C the total coliform count increases from 2.78034 log CFU/ml to 2.95061 log CFU/ml. The minimum growth of total coliform count 2.78034 log CFU/ml was observed at 25 ^{0}C, 6 hr, 22.5 PPM sodium thiocyanate, and 15 PPM hydrogen peroxide concentration. The total coliform count was inversely related to storage temperature i.e. when the storage temperature increases the growth of total coliform count would be increased this was due to the growth of coliform bacteria accelerated at high temperature. Therefore for coliform bacteria that survive the initial bactericidal activity of the LPs, their recovery period to regular growth is highly temperature-dependent, being much faster at high temperatures

than lower temperature (Kamau and Kroger 1984). This implies that the growth of coliform directly dependent on the storage temperature (Srisaikham et al., 2017).

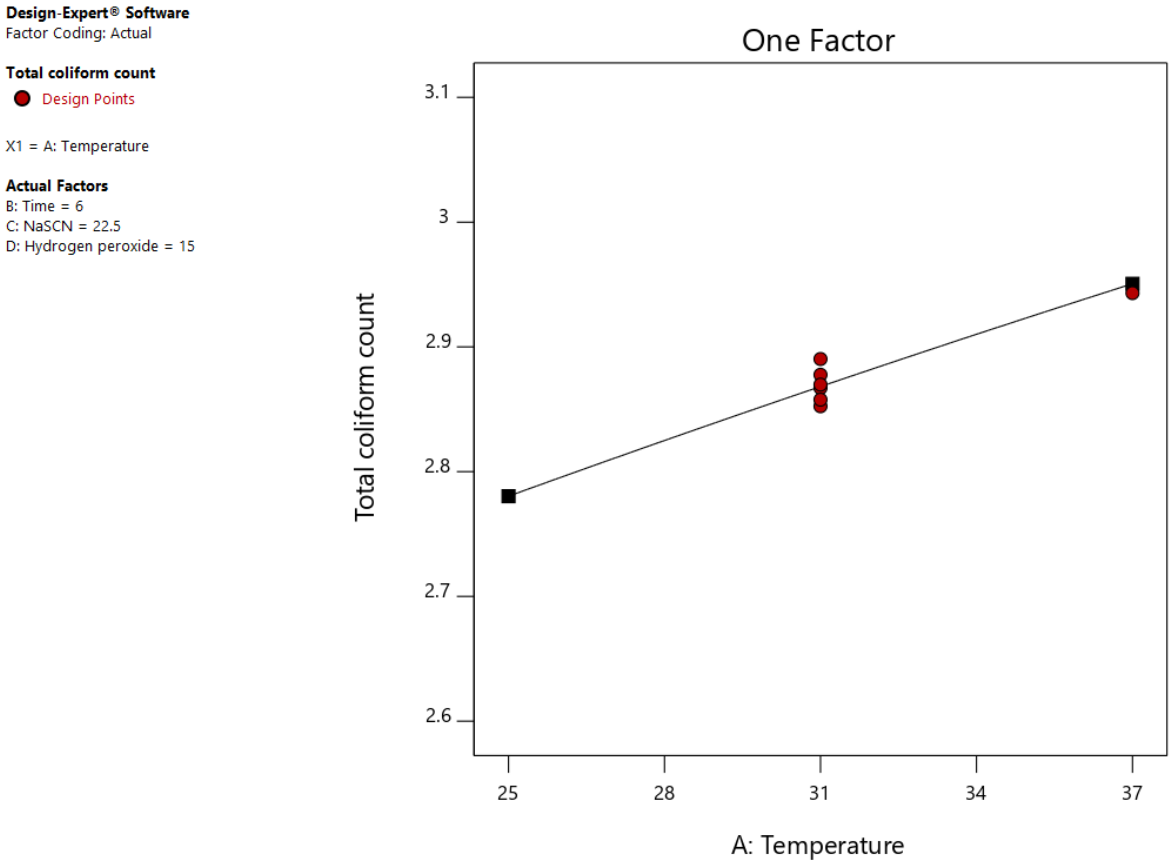

Figure 4.4 Effect of temperature on the of total coliform count

4.6.2 Effect of time on total coliform count

Storage time is known to play a significant role in the growth of total coliform count in lactoperoxidase activated goat milk. The effect of increasing and decreasing time on total coliform count has been shown in Figure 4.5; the effect of time was varied between the intervals ranges from 4 hr to 8 hr with an increase in the total coliform count from 2.78026 log CFU/ml to log CFU/ml 2.95787. The minimum growth of total coliform count 2.78026 log CFU/ml was obtained at 4 hr of storage time with the operating conditions of 31 ^{o}C, 22.5 PPM sodium thiocyanate, and 15 PPM hydrogen peroxide concentration. The total coliform count was directly related to storage time i.e. as storage time increase the growth of total coliform count increase while the total coliform count decrease with decreasing storage time. The reason is that the antibacterial products hypothiocyanate ion and hypothiocyanous acid are instable and short lived products, as time increase their availability decrease and results in

decrease the effectiveness of antimicrobial. Therefore the efficacy of the LPs perseveres for a limited period of time (Althaus et al., 2001).

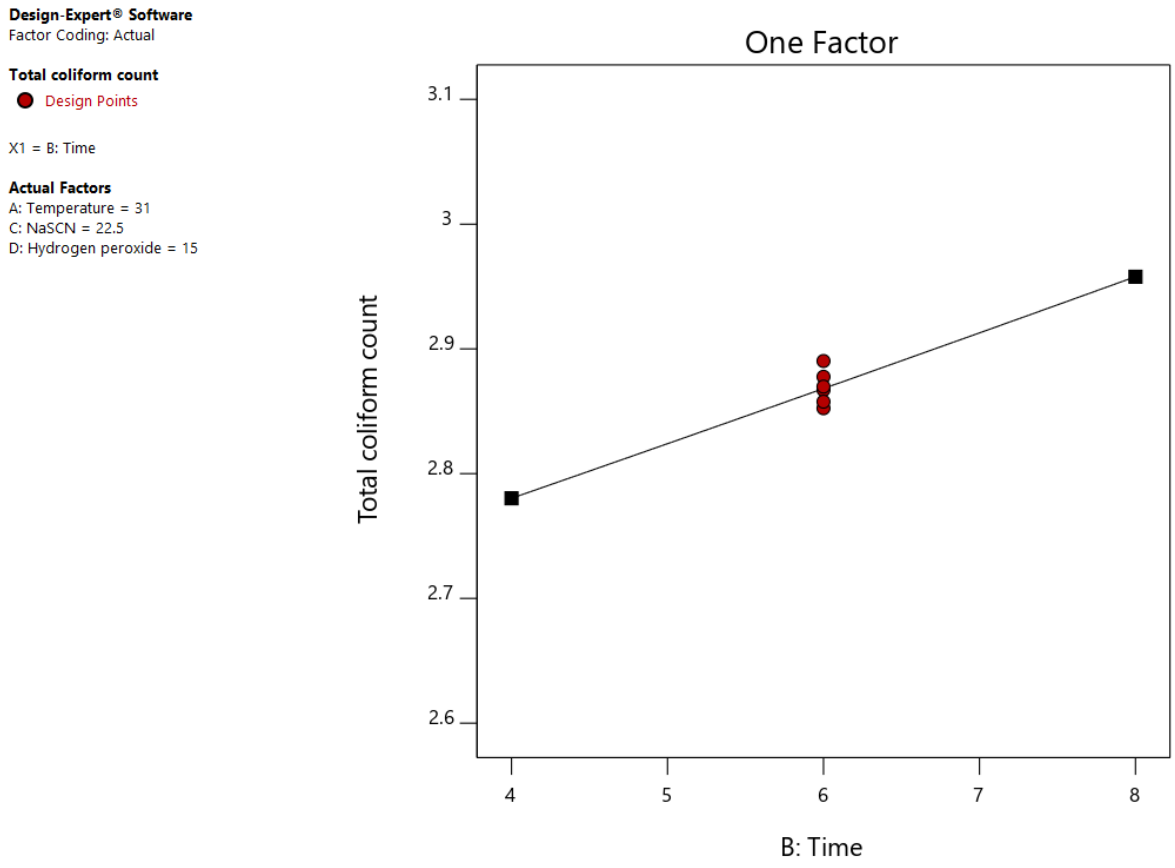

Figure 4.5 Effect of time on total coliform count.

4.6.3 Effect of sodium thiocyanate (NaSCN) on total coliform count

The effect of sodium thiocyanate on the growth of total coliform count was studied using the plot in Figure 4.6. The effect of sodium thiocyanate on the activation of lactoperoxidase system was varying in ranges from 15 PPM to 30 PPM was studied by maintaining other parameters as constant storage temperature 31 ^{0}C, storage time 6 hr, and 15 PPM hydrogen peroxide. As the sodium thiocyanate was increased from 15 PPM to 30 PPM, the growth of total coliform count was found to be decreased from 2.90439 log CFU/ml to 2.83042 log CFU/ml.

When sodium thiocyanate concentration (as a source of thiocyanate ion) increases, the formation antibacterial products i.e. hypothiocyanate ion ($OSCN^-$) and hypocyanous acid ($HOSCN$) would be increased due to the oxidation of the high amount of thiocyanate ion (SCN^-). Even though the lactoperoxidase enzyme present in milk is in resting (not

active). It reacts with hydrogen peroxide and changes to compound *I* (ferric peroxidase enzyme) state. When low thiocyanate ion is present compound *I* reacts with hydrogen peroxide and the enzyme reduced to the ground state. However, when high amount of thiocyanate available the activity of lactoperoxidase enzyme increases, and results in to increase the antibacterial product formation (Seifu et al., 2005).

According to Panthanara et al., (2005) the thiocyanate ion (SCN^-) in raw milk increased with increasing sodium thiocyanate (NaSCN) concentration. Similarly, Srisaikham et al., (2017) reported that at the highest sodium thiocyanate and sodium percarbonate (as source of hydrogen peroxide) ratio the highest thiocyanate (SCN^-) ion and LP activity were observed.

However, excessive intake of thiocyanate ion (SCN^-) may have toxic effect which is not quite unique with thiocyanate ion (SCN^-) only because excess of even vitamins could be potentially toxic. Therefore excessive intake of thiocyanate ion (SCN^-) may cause disturbances in the thyroid function indirectly through interference with iodine metabolism. It has been demonstrated that doses between 200 - 400 PPM of thiocyanate ion (SCN^-) are necessary to cause a thyrostatic effect and reduction in iodine uptake (Kumar 1986; WHO 2005). This dose of 200-400 PPM would be equivalent to an intake 6.67 to 13.33 litre of milk containing 30 ppm thiocyanate ion (SCN^-) which would be practically impossible.

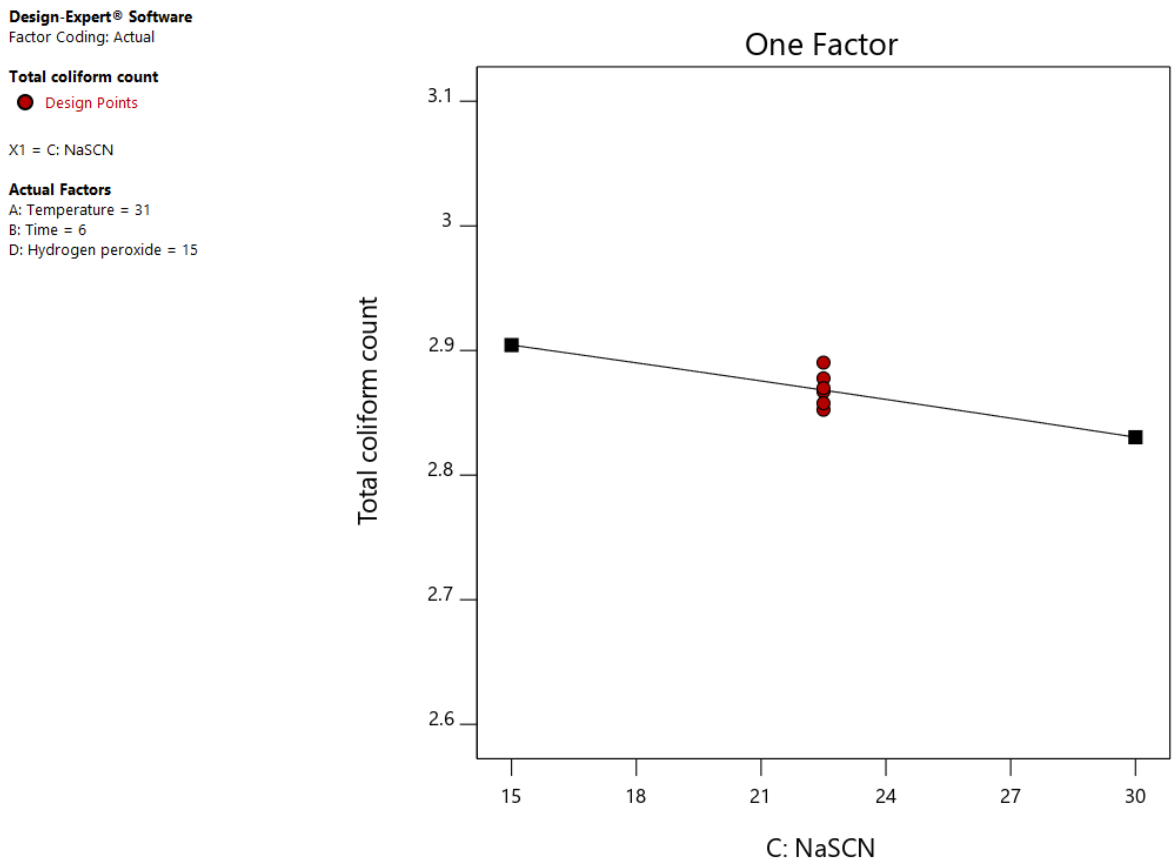

Figure 4.6 Effect of NaSCN concentrations on total coliform count

4.6.4 Effect of hydrogen peroxide (H_2O_2) on total coliform count

Hydrogen peroxide is known to play a significant role in the growth of total coliform count. The effect of increasing and decreasing hydrogen peroxide on the growth of total coliform count in goat milk has been shown in Figure 4.7; the effect of hydrogen peroxide was varied between the intervals ranges from 10 PPM to 20 PPM mm with an decrease in the growth of total coliform count from 2.90236 log CFU/ml to 2.87407 log CFU/ml. The minimum growth of total coliform count 2.86709 log CFU/ml was obtained at 15 PPM of hydrogen peroxide with the operating conditions of 31 ^{0}C storage temperature, 6 hr storage time, and 22.5 PPM sodium thiocyanate concentration.

According to Srisaikham et al., (2017) at the highest sodium thiocyanate and sodium percarbonate (source of hydrogen peroxide) ratio the highest thiocyanate ion (SCN^-) and LP activity was reported. But, in the presence of an excess of hydrogen peroxide and low amount of thiocyanate ion(SCN^-) results in the formation of Compound III, which is associated with irreversible inactivation of lactoperoxidase enzyme (Seifu et al., 2005). Even also when H_20_2 was in excess of thiocyanate ion(SCN^-), the yield of hypothiocyanate ion ($OSCN^-$) was

lower than the amount of thiocyanate ion(SCN^-), suggesting that hypothiocyanate ion ($OSCN^-$) which is the antibacterial product was oxidized by the excess H_20_2 (Gupta 1985).

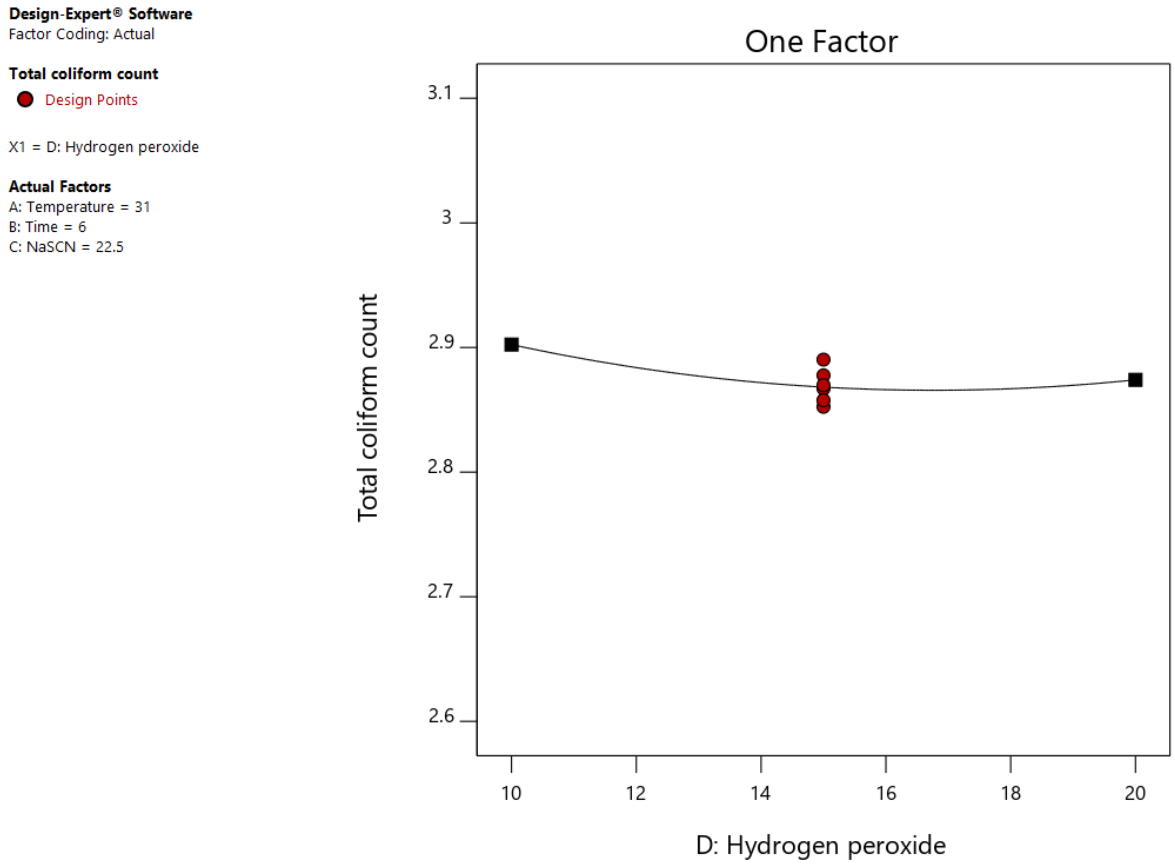

Figure 4.7 Effect of hydrogen peroxide on total coliform count

4.7 The interaction effect of process parameters on the total coliform count

Figure 4.8 shows us the interaction effect of temperature and time on the total coliform count at a fixed sodium thiocyanate and hydrogen peroxide. As it was shown in figure 4.8 the temperature and time have a high effect on the total coliform count. As the interaction effect of temperature and time increases the total coliform count also increases, this was due to the instability and short lived antibacterial products, as time increase the availability and antibacterial property decrease. At this condition when there is a high temperature the growth of total coliform bacteria increased rapidly. Even though for coliform bacteria that survive the initial bactericidal activity of the LPs they restart to recover their damaged parts when they found optimum temperature or high temperature (Kamau and Kroger 1984). Then the coliform bacteria resume their regular growth after recovery, and with increasing time and temperature the growth total coliform bacteria increase. Like the individual effect, when

temperature and time interact jointly the total coliform count increases as the interaction of temperature and time increases, at high temperature and time the total coliform count is high, and at low temperature and time the total coliform count is low. Due to this operating at lower time and lower temperature with center point sodium thiocyanate and hydrogen peroxide is optimum.

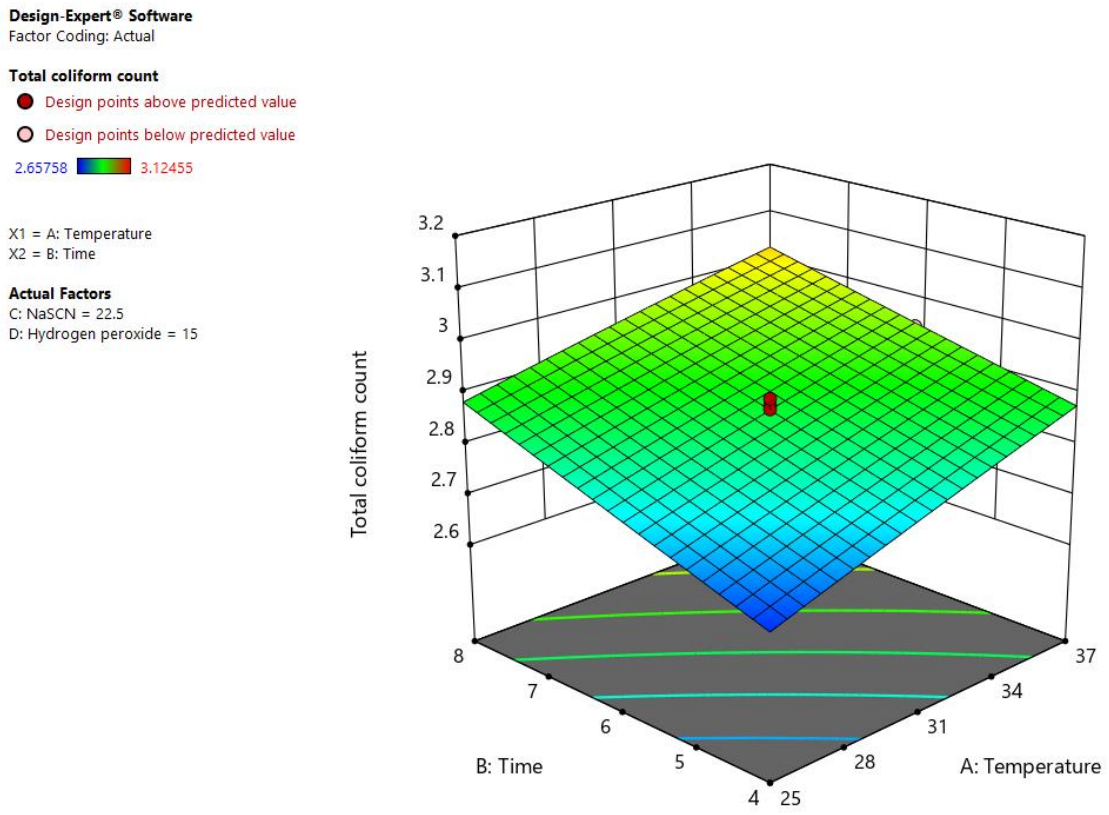

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Figure 4.8: 3D surface showing effect of time and temperature on the TCC

Figure 4.9 shows us the interaction effect of time and sodium thiocyanate (NaSCN) concentration on the total coliform count at fixed temperature and hydrogen peroxide (H_2O_2). Time and sodium thiocyanate concentration has a high effect on the total coliform count. As the time decrease and sodium thiocyanate increase, the growth of total coliform count decrease this was due to the antibacterial products i.e. hypothiocyanate and hypocyanous acid produced by the oxidation of thiocyanate ion depends on the level of thiocyanate ion

(SCN^-), with increasing of thiocyanate ion the formation of antibacterial products increase (Reiter et al., 1976). However the antibacterial products are instable and short lived they decrease with increasing of time. Therefore the minimum growth of total coliform was obtained at minimum time and maximum sodium thiocyanate concentration which is 4 hr and 30 PPM sodium thiocyanate concentration.

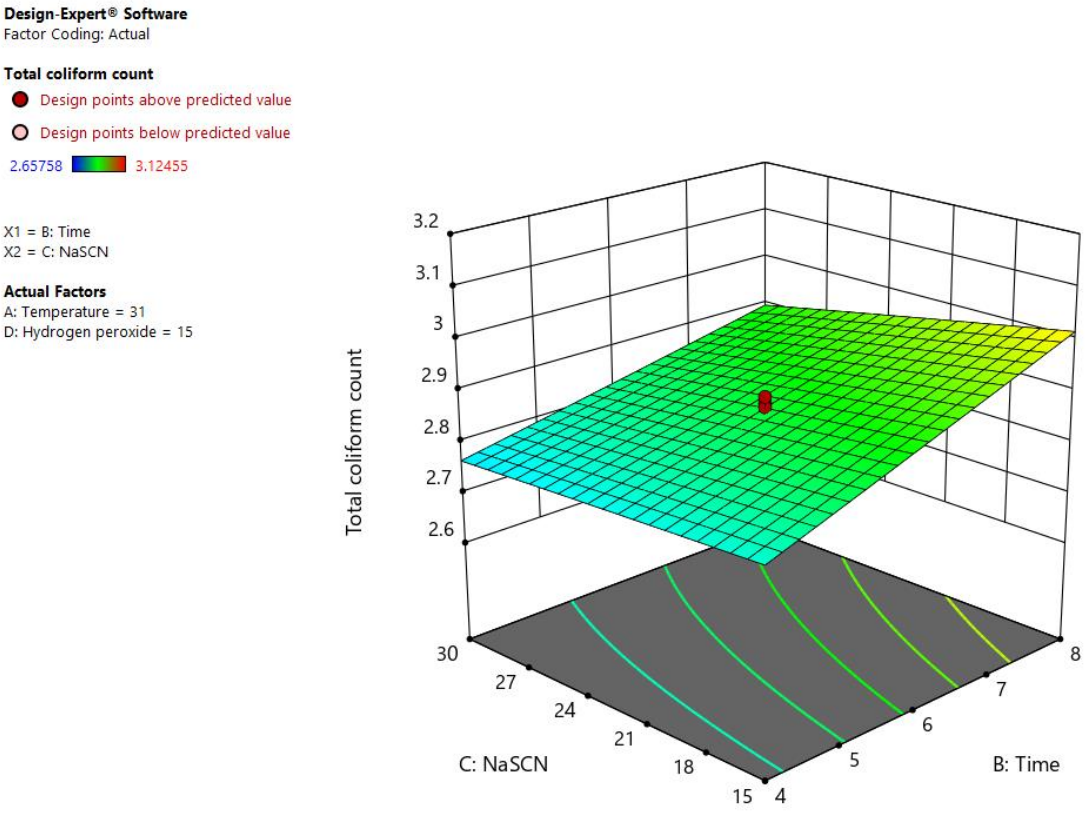

Figure 4.9 3D surface showing effects of time and NaSCN on the TCC

Figure 4.10 shows us the interaction effect of sodium thiocyanate and hydrogen peroxide (H_2O_2) concentration on the growth of total coliform count at fixed time and temperature. As it was shown in figure 4.10, sodium thiocyanate and hydrogen peroxide concentration have an effect on the growth of total coliform count. As sodium thiocyanate and hydrogen peroxide concentration increased the growth of total coliform count decrease while with decreasing sodium thiocyanate and hydrogen peroxide the growth of total coliform count increase. According to Srisaikham et al., (2017) at the highest sodium thiocyanate and sodium percarbonate (as source of hydrogen peroxide) ratio the highest thiocyanate ion (SCN^-) and LP activity were observed. The results showed that the LP system appeared to

have the best antagonistic activity with the highest thiocyanate ion and hydrogen peroxide concentrations. This might be due to high concentrations of thiocyanate ion and hydrogen peroxide the production of antibacterial product would be increased, results in reduction of the acid development and increasing the keeping quality of the milk (Boulares 2011).

Therefore minimum growth of total coliform 2.81958 log CFU/ml was obtained at the higher ratio of sodium thiocyanate and hydrogen peroxide, which is 30 PPM of thiocyanate and 20 PPM of hydrogen peroxide concentration.

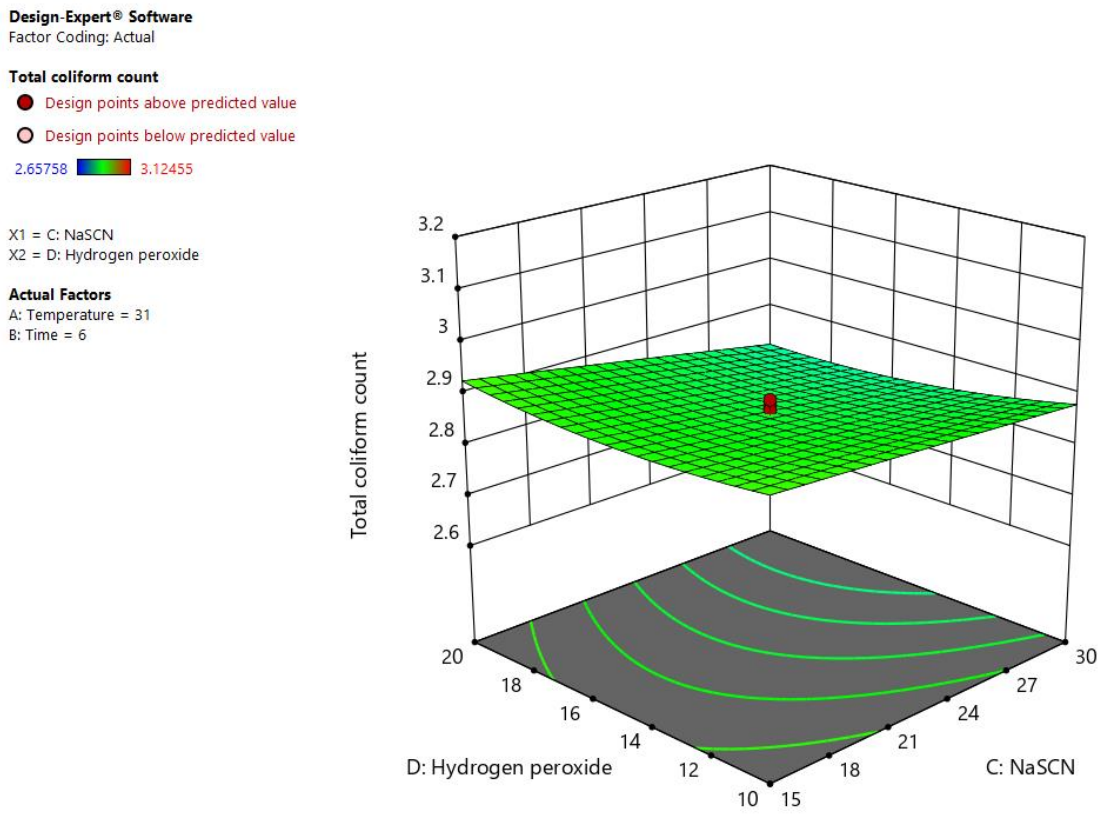

Figure 4.10 3D surface showing effect of NaSCN and H_2O_2 on the TCC

4.8 Optimizations

The result in Table 4.4 shows that the four process variables affected the growth of total coliform count. Therefore, the next step was to optimize the process variables in order to obtain the minimum growth of total coliform count by using the model regression developed. So, in order to obtain the minimum growth of total coliform count, using optimization function in Design-Expert 11.0 software, it was predicated that at the following operating condition; 25^0C storage temperature, 4 hr storage time, 29.98 PPM of sodium thiocyanate,

16.603 PPM of hydrogen peroxide, a minimum growth total coliform count 2.658 log CFU/ml was obtained.

To validate the optimum conditions predicted by the model using desirability ramp, triplicate experiments were conducted using the optimized process conditions and mean growth of total coliform count 2.665 log CFU/ml was obtained and the experiment results were related with the data obtained from optimization analysis using desirability functions. From design expert soft-ware of numerical optimization tool, the optimized solutions for minimum growth of total coliform count is shown in Table 4.7.

Table 4.6 Optimization criteria for optimum growth of TCC

Name	Goal	Lower Limit	Upper Limit
Temperature (^{o}C)	In range	25	37
Time (hr)	In range	4	8
Sodium Thiocyanate(PPM)	In range	15	30
Hydrogen peroxide (PPM)	In range	10	20
Total coliform count(CFU/ml)	Minimize	2.65758	3.12455

Table 4.7 Solution output from numerical optimization

Number	Temperature	Time	NaSCN	Hydrogen peroxide	Total coliform count	Desirability	
1	25.019	4.006	29.980	16.603	2.658	1.000	Selected
2	25.047	4.109	29.721	18.739	2.657	1.000	
3	25.107	4.009	28.914	19.985	2.657	1.000	
4	25.000	4.233	29.998	18.347	2.660	0.995	
5	25.445	4.000	29.519	20.000	2.660	0.994	
6	25.000	4.001	27.612	17.375	2.660	0.994	
7	25.305	4.140	30.000	20.000	2.661	0.993	
8	25.000	4.000	30.000	15.803	2.661	0.992	

The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If a response falls within the unacceptable intervals, the desirability is 0, and if a response falls within the ideal intervals or the response reaches its ideal value, the desirability is 1.

4.9 Major chemical composition of raw and LP activated goat milk

The preserved goat milk was analyzed for some selected microbiological and physicochemical properties so as to determine the quality of the preserved milk at the optimum parametric condition of 29.98 PPM NaSCN, 16.6 PPM H_2O_2 and 25^oC.

Table 4.8 Major chemical composition of raw and LP activated goat milk (mean ± SD)

Item	Time (Hr)	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	TS (%)
Raw milk	0	$84.8^A \pm 0.06$	$3.88^B \pm 0.02$	$5.65^C \pm 0.03$	$0.88^D \pm 0.02$	$15.2^E \pm 0.06$
Preserved milk	10	$85.02^A \pm 0.02$	$3.84^B \pm 0.04$	$5.61^C \pm 0.02$	$0.85^D \pm 0.02$	$14.98^E \pm 0.02$
P-value		0.209	0.128	0.132	0.249	0.261

A, B, C, D, and E Means within the same column within time having different superscript letters are different at P<0.05 SD = standard deviation, TS = total solids.

Data in Table 4.8 represent the chemical composition of the activated LP goat's milk samples and the control stored at 25^oC temperature. The results indicated that the values of moisture content, protein, fat, total solids, and ash in all raw milk samples and preserved milk samples had no significant (P>0.05) differences. Means that do not share a similar letter with in the same column are significantly different. Therefore the raw milk and preserved milk by lactoperoxidase system they don't have any significant difference on their chemical composition this was due to the antibacterial products of the lactoperoxidase system which kills or inhibit the growth of microbes and keeps the quality of the milk.

Azeze (2015) reported that the mean total solid, fat, protein, lactose, ash and moisture content of southern Ethiopia goat milk to be 13.57, 4.91, 3.48, 4.81, 0.4 and 86.42%, respectively. Gaddour et al., (2013) also reported that goat milk has 3.963, 3.13 and 0.78% of fat, protein, and ash, respectively. The results from Table 4.8 are slightly different from these reports due to different reasons such as season, stages of lactation, breed of the goats, diet, and environmental conditions (Haenlein 2004).

Table 4.9 lactose and SNF composition of raw and LP activated goat milk (mean ± SD)

Item	Lactose %	SNF%
Raw goat milk (0 hr)	$4.79^{A} \pm 0.1$	$9.55^{B} \pm 0.08$
Preserved goat milk (10 hr)	$4.68^{A} \pm 0.03$	$9.37^{B} \pm 0.03$
P-value	0.439	0.209

A and B Means within the same column within time having different superscript letters are different at P<0.05 SD = standard deviation.

Data in Table 4.9 represent the chemical composition of the activated LP goat's milk samples and the control stored at 25°C temperature. The results indicated that the values of lactose content and SNF in all milk samples had no significant (P>0.05) differences.

Acidity test

Acidity of the milk sample was determined by continuous titration of the milk by alkaline solution until a definite pink color was observed

The acid content of the milk was determined using equation 3.3

Milk sample taken – 10 ml

Milliliters of alkali taken for titration – 1.9 ml then using equation 3.3

$$Lactic\ \text{acid}(\%) = \frac{ml\ \text{alkali} \times 0.009 \times 100}{ml\ \text{sample}} = \frac{1.9\ ml \times 0.009 \times 100}{10\ \text{ml}} = 0.171\ \%$$

The same procedure was done after 10 hr for preserved goat milk by sodium thiocyanate and hydrogen peroxide at 25°C storage temperature

Milk sample taken – 10 ml

Milliliters of alkali taken for titration – 2.3 ml

$$Lactic\ \text{acid}(\%) = \frac{ml\ \text{alkali} \times 0.009 \times 100}{ml\ \text{sample}} = \frac{2.3\ ml \times 0.009 \times 100}{10\ \text{ml}} = 2.07\ \%$$

Table 4.10 Titratable acidity of raw and treated goat's milk

Treatment	Storage time (hr)	
	Initial	10
Raw goat milk	$0.173^{C} \pm 0.001$	$0.328^{A} \pm 0.003$
Preserved goat milk	$0.173^{C} \pm 0.001$	$0.204^{B} \pm 0.006$
P-value		
Time	0.000	
Milk	0.000	
Time * Milk	0.000	

A, B, C Means within the same column within time having different superscript letters are different at P<0.05 SD = standard deviation.

Results in Table 4.10 represent the titratable acidity (expressed as lactic acid %) of goat milk treated with sodium thiocyanate and hydrogen peroxide kept at incubator (25^{o} C) for 10 hrs. The results demonstrated that there was a significant (P<0.05) improvement in the quality of the treated milk samples at 25°C storage. The data indicated that the storage period had significant (P < 0.05) effect on the titratable acidity of the milk samples. As the storage period increased the titratable acidity increased but the quality of the milk samples was not deteriorated until the 10 hr (0.204 ±.006%) of the LP activated milk while the titratable acidity of the control was high at 10 hr (0.328±.01%) which indicated deterioration in the quality of the milk samples. Therefore the results showed that there were significant variations in the titratable acidity of the control samples and the treated. The development of acid by the conversion of lactose is low in the preserved milk due to the inhibition of bacteria growth by the lactoperoxidase system. However for the control the acid development is high due to the bacteria growth and converts the lactose to lactic acid.

The value for the titratable acidity of raw goat milk samples obtained from current study was 0.173 % lactic acid which was slightly higher than the reported values of 0.135 % by (Imran et al., 2008; Mayer and Fiechter 2012; Soliman 2005) but lower than 0.21% reported by (Nigussie & Seifu 2008). The acidity of milk samples varies due to carbon dioxide, phosphates, citrates, caseins and whey proteins in the milk (Lai et al., 2016). It could be

varied also due to factors of lactation period where it was frequently having lower acidity towards the end of the lactation stage (Lai et al. 2016).

The acid content of the preserved milk after 10 hr was 0.204 % which is in an acceptable range for the goat milk and could be used for further processing. The delay in acid development observed until 10 hr of storage in LP activated goat milk samples indicates that under the current condition, activation of the LP system can keep goats' milk fresh for up to 10 hr during storage at 25^0C. This result is in line with the findings of Haddadin et al., (1 996) reported that acidity of LP-treated goat milk samples held at 22°C is stable for 9 - 1 2 hr.

Table 4.11 Effect of NaSCN and H_2O_2 on TBC

Total bacterial count Log CFU/ml(mean ± SD)				
Time (hr)	0	4	8	12
Raw goat milk	4.06^{E} ± 0.02	4.65^{D} ± 0.006	5.02^{C} ± 0.02	7.15^{A} ± 0.02
Preserved goat milk	4.05^{E} ± 0.02	3.83^{F} ± 0.02	4.03^{E} ± 0.02	5.68^{B} ± 0.02
P-value				
Time	0.000			
Milk	0.000			
Time * Milk	0.000			

A, B, C, D, E, and F Means within the same column and raw within time having different superscript letters are different at P<0.05 SD = standard deviation.

Data in Table 4.11 represent mean total bacterial counts (TBC) of raw goat's milk samples stored at 25°C. The results showed that there were significant ($p<0.05$) differences in the mean total bacterial counts (TBC) of treated raw goat's milk samples and the control throughout the storage period. The highest TBC was (7.15 ± 0.02 log CFU/ml) at 12 hr while the lowest one was (3.83 ± 0.02 log CFU/ml) for the treatment milk sample at 4 hr. The results showed that as the storage times increased the TBC increased and reached the highest value.

The initial bacterial count of our result seemed to be slightly higher than 4.0 log CFU/ml reported by (Bhat and Nadiah 2012) but lower than 7.07 log CFU/ml reported by (Nigussie and Seifu 2008). The bacterial load might be varied due to different reasons such as infected udder of goat, unhygienic milking procedure, poor water quality used for cleaning, use of

unsterilized equipment's and milk storage conditions are considered some of the common factors responsible for variation (Chye *et al*., 2004; Suguna *et al*., 2011).

According to Cempirkova (2002), in Europe, milk qualities are monitored based on the presence of total bacterial count, which should not exceed 4.5 log CFU/ml. While in the United States, bacterial count in goat milk is allowed up to 5.0 log CFU/ml. The limitation in goat milk is the same as that of cow milk (Zweifel *et al*., 2005).

In the present study, the total plate count of preserved goat milk at 10 hr was 4.51 log CFU/ml. Based on this, our results indicate that bacteria load recorded in preserved goat milk samples up to 10 hr are still considered to be at a safe range.

Table 4.12 Effect of NaSCN and H_2O_2 on TCC

Total coliform count Log CFU/ml(mean ± *SD)*				
Time (hr)	0	4	8	12
Raw goat milk	2.77^{E} ± 0.03	3.01^{D} ± 0.01	3.94^{C} ± 0.03	5.49^{A} ± 0.01
Preserved goat milk	2.77^{E} ± 0.02	2.66^{F} ± 0.002	2.79^{E} ± 0.03	4.13^{B} ± 0.02
P-value				
Time	0.000			
Milk	0.000			
Time * Milk	0.000			

A, B, C, D, E, and F Means within the same column and raw within time having different superscript letters are different at P<0.05 SD = standard deviation.

Data in Table 4.12 represent the mean total coliform count (TCC) of raw goat's milk samples stored at 25°C. Table 4.12 showed that there were significant (p<0.05) differences in the mean total coliform count (TCC) of treated raw goat's milk samples and the control throughout the storage period. The highest TCC was (5.49 ± 0.01 log CFU/ml) at 12 hr while the lowest one was (2.66 ± 0.002 log CFU/ml) for the treatment milk sample at 4 hr. The results showed that as the storage times increased the TCC increased and reached the highest value.

European Council, (1992) has stipulated that Coliforms count should be no more than 2.0 log CFU/ml goat's milk. From the above-mentioned criteria, our findings concerning the microbiological examination of goat's milk samples exceed these standards. However higher findings also reported by (Bhat and Nadiah 2012) 2.2 and 4.0 log CFU/ml on farm 1 and farm 2 respectively and 5.63 log CFU/ml reported by (Nigussie and Seifu 2008). The present study shows that the TCC of goat's milk samples exceeds the European limit but there is no definite limit in Ethiopia Standards.

Table 4.13 The result summary on microbiology and physiochemical properties of goat milk

Test	Experimental results		
	Raw goat milk	Preserved goat milk	Literature
Moisture content	84.8	85.02	84.45 - 88.87
Protein content	3.88	3.84	3.04 – 3.69
Fat content	5.65	5.61	3.06 – 5.80
Ash content	0.88	0.85	0.4 – 0.8
Total solids	15.2	14.98	11.13 – 15.55
Lactose content	4.79	4.68	4.63 – 5.21
Acid content	0.173	0.204	0.135 – 0.21
pH	6.6	6.52	6.5 – 6.7
Freezing point	-0.52		(-0.52) – (- 0.56)
Total coliform count (log CFU/ml)	2.77	3.17	1.87 - 4
Total bacterial count (log CFU/ml)	4.06	4.51	3.44 – 7.07

(Sawaya 1984; Cemprikova 2002; Haenlein 2004; Soliman 2005; Park 2007; Imran et al., 2008; Nigussie and Seifu 2008; Bhat and Nadiah 2012; Mayer and Fiechter 2012; Suguna 2012; Gaddour et al., 2013; Azeze 2015)

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In the present research work, a parametric study of the activation of the lactoperoxidase system, optimization and characterization of raw and preserved goat milk was undertaken based on central composite design CCD using Design-Expert software. The activation of the lactoperoxidase system was done using temperature (25 – 37)^{o}C, storage time (4 – 8) hr, sodium thiocyanate (15 – 30)PPM and hydrogen peroxide (10 – 20)PPM were the considered parameters for optimization investigation.

The optimum set of operational conditions from the experimental process was obtained that: at 25 ^{o}C temperature, 4 hr of storage time, 29.98 PPM of sodium thiocyanate and 16.6 PPM of hydrogen peroxide. The minimum growth of total coliform count at these optimum operational conditions was found to be 2.658 log CFU/ml.

From design expert software the analysis of ANOVA ($P < 0.05$) for storage temperature, storage time, sodium thiocyanate and hydrogen peroxide concentration indicated that operating parameters have significant effect on the growth of total coliform count. It was clearly seen that storage temperature, storage time and sodium thiocyanate concentration are the most significant ($P < 0.0001$) effect on the growth of total coliform count, since Probability Values of "Prob > F values" indicate model terms are highly significant.

The microbiological analysis and physicochemical properties of the raw and preserved goat milk like total bacteria, total coliform count, protein content, fat content, lactose content, ash content, and total solids were determined and obtained comparable results from literatures. The lactoperoxidase activated goat milk keeps the quality of goat milk up to 10 hr but for the control up to 4 hr. Even the LPs do not induce any significant adverse effects on the physicochemical characteristics of raw goat milk. Therefore it is very important to have more research on goat milk and processed products of goat milk (i.e. yoghurt and cheese) which are preserved by lactoperoxidase system in the future to explore the potential effects of lactoperoxidase system in preserving and keeping the quality for a long time without any adverse effects.

5.2 Recommendations

The following recommendations were drawn from this thesis work for future work as:

- Alternative methods of extending shelf life of goat milk using lysozyme and lactoferrin need to be done in order to investigate the variation that could arise on the quality and growth of the microbes as a result of using different methods LPs activation.
- It is also, recommend that in this study LPs variables are optimized; future studies should include optimization of LPs in combination with other conventional preservation treatments to inhibit pathogenic microorganisms
- Further investigation should be done to analyze the effects of LPs and extend the shelf life of the processed goat milk such as cheese and yoghurt
- Other specific spoilage microbial should be identified to investigate the effectiveness of the lactoperoxidase system in keeping the quality of milk
- It is recommended that the concerned bodies should raise awareness on the importance of the system and extends the result of this study in the country for improving the preservation and reducing milk loss due to microbial spoilage.

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Appendix

Appendix A: Central Composite design matrix and experimental results

Run NO	Factor 1 Temperature (^{o}c)	Factor 2 Time (Hr)	Factor 3 NaSCN (PPM)	Factor 4 H_2O_2 (PPM)	TCC CFU/ml	TCC Log CFU/ml
1	25	8	30	10	7.636×10^2	2.88289
2	37	8	15	20	1.33×10^3	3.12455
3	31	6	22.5	15	7.206×10^2	2.85769
4	25	8	15	20	8.954×10^2	2.95204
5	37	8	30	20	9.202×10^2	2.96387
6	31	6	22.5	15	7.409×10^2	2.86976
7	31	6	22.5	25	8.364×10^2	2.9224
8	37	8	15	10	1.190×10^3	3.07571
9	37	8	30	10	1.05×10^3	3.02119
10	31	6	22.5	15	7.545×10^2	2.87769
11	25	8	15	10	9.249×10^2	2.96613
12	25	4	30	10	5.136×10^2	2.71066
13	37	4	15	10	8.239×10^2	2.91585
14	37	4	30	10	7.545×10^2	2.87769
15	25	4	15	20	4.898×10^2	2.69005
16	31	6	22.5	15	7.364×10^2	2.86709
17	25	4	30	20	4.545×10^2	2.65758
18	31	6	22.5	15	7.768×10^2	2.89029
19	19	6	22.5	15	5.127×10^2	2.7099
20	31	6	22.5	5	9.887×10^2	2.99507
21	25	8	30	20	6.108×10^2	2.78591
22	37	6	22.5	15	8.773×10^2	2.94313
23	31	10	22.5	15	1.121×10^3	3.04978
24	37	4	30	20	7.152×10^2	2.8544
25	37	4	15	20	8.249×10^2	2.91643
26	31	6	22.5	15	7.119×10^2	2.85242
27	31	6	37.5	15	6.273×10^2	2.79746
28	31	2	22.5	15	5.182×10^2	2.71448
29	31	6	7.5	15	8.986×10^2	2.95355
30	25	4	15	10	4.879×10^2	2.68888

Appendix B: Diagnostic report of actual, predict and residual

Run Order	Standard Order	Actual Value	Predicted Value	Residual
1	7	2.88	2.89	-0.0062
2	12	3.12	3.12	0.0083
3	30	2.86	2.87	-0.0105
4	11	2.95	2.95	0.0020
5	16	2.96	2.96	0.0061
6	27	2.87	2.87	0.0015
7	24	2.92	2.92	0.0025
8	4	3.08	3.10	-0.0246
9	8	3.02	3.01	0.0126
10	26	2.88	2.87	0.0095
11	3	2.97	2.97	-0.0008
12	5	2.71	2.72	-0.0121
13	2	2.92	2.90	0.0156
14	6	2.88	2.90	-0.0193
15	9	2.69	2.71	-0.0164
16	25	2.87	2.87	-0.0011
17	13	2.66	2.65	0.0074
18	28	2.89	2.87	0.0221
19	17	2.71	2.69	0.0229
20	23	3.00	2.98	0.0186
21	15	2.79	2.81	-0.0194
22	18	2.94	2.95	-0.0075
23	20	3.05	3.05	0.0006
24	14	2.85	2.86	-0.0030
25	10	2.92	2.93	-0.0110
26	29	2.85	2.87	-0.0158
27	22	2.80	2.79	0.0065
28	19	2.71	2.69	0.0205
29	21	2.95	2.94	0.0146
30	1	2.69	2.71	-0.0233

Appendix C: Fit summary

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	248.74	1	248.74			
Linear vs Mean	0.3846	4	0.0962	70.70	< 0.0001	
2FI vs Linear	0.0167	6	0.0028	3.05	0.0292	
Quadratic vs 2FI	0.0117	4	0.0029	7.86	0.0013	Suggested
Cubic vs Quadratic	0.0045	9	0.0005	2.68	0.1216	Aliased
Residual	0.0011	6	0.0002			
Total	249.16	30	8.31			

Appendix D: The regression coefficient estimate of the process variable

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.87	1	0.0076	2.85	2.88	
A-Temperature	0.0851	1	0.0045	0.0756	0.0946	1.12
B-Time	0.0888	1	0.0039	0.0804	0.0972	1.0000
C-NaSCN	-0.0370	1	0.0039	-0.0454	-0.0286	1.0000
D-Hydrogen peroxide	-0.0141	1	0.0039	-0.0225	-0.0057	1.0000
AB	-0.0137	1	0.0048	-0.0240	-0.0034	1.0000
AC	-0.0035	1	0.0048	-0.0138	0.0068	1.0000
AD	0.0082	1	0.0048	-0.0021	0.0185	1.0000
BC	-0.0221	1	0.0048	-0.0324	-0.0118	1.0000
BD	-0.0028	1	0.0048	-0.0131	0.0075	1.0000
CD	-0.0167	1	0.0048	-0.0270	-0.0064	1.0000
A^2	-0.0027	1	0.0048	-0.0129	0.0074	1.12
B^2	0.0008	1	0.0037	-0.0069	0.0086	1.03
C^2	-0.0008	1	0.0037	-0.0086	0.0070	1.03
D^2	0.0200	1	0.0037	0.0122	0.0278	1.03

Appendix E: Laboratory work picture

E.1 goats

E.2 raw goat milk sample

E.3 ice box

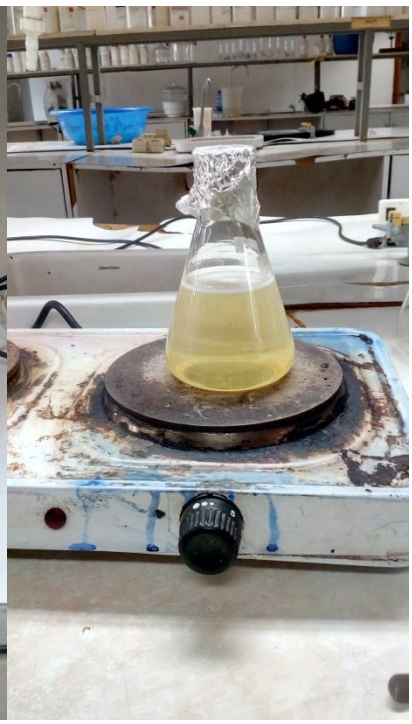
E.4 raw goat milk for analysis

E.5 preserved goat milk

E.6 freshness analysis of raw goat milk

E.7 test tube with peptone

E.8 violet red bile agar

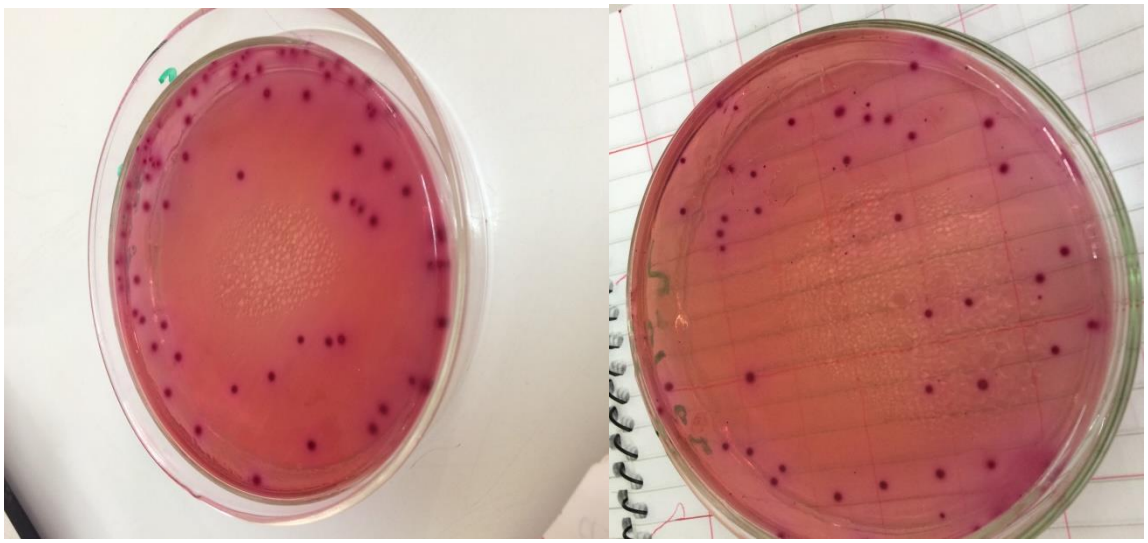


E.9 plate count agar

E.10 safety cabinet

E.11 autoclave

E.12 incubator



E.13 coliform bacteria and precipitated preserved milk