THE DEVELOPMENT OF ALKALINE PHOSPHATASE BASED PAPER BIOREPORTER FOR EVALUATION OF MILK PASTEURIZATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

CEREN KARAKAŞ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JUNE 2009

Approval of the thesis:

THE DEVELOPMENT OF ALKALINE PHOSPHATASE BASED PAPER BIOREPORTER FOR EVALUATION OF MILK PASTEURIZATION

submitted by **CEREN KARAKAŞ** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University by,**

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Gülay Özcengiz
Head of Department, **Biotechnology** _____

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biology Dept., METU** _____

Dr.Can Özen
Co-Supervisor, **Central Lab.R&D Center, METU** _____

Examining Committee Members:

Prof. Dr. Meral Yücel
Biology Dept., METU _____

Prof. Dr. Hüseyin Avni Öktem
Biology Dept., METU _____

Prof. Dr. Haluk Hamamcı
Food Engineering Dept., METU _____

Prof. Dr. Ufuk Bakır
Chemical Engineering Dept., METU _____

Assoc. Prof. Dr. Füsun İnci Eyidoğan
Education Faculty, Başkent University _____

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name :

Signature :

ABSTRACT

THE DEVELOPMENT OF ALKALINE PHOSPHATASE BASED PAPER BIOREPORTER FOR EVALUATION OF MILK PASTEURIZATION

Karakaş, Ceren

M.S., Department of Biotechnology

Supervisor : Prof. Dr. Hüseyin Avni Öktem

Co-supervisor: Dr. Can Özen

June 2009, 148 pages

Alkaline phosphatase (ALP) is a natural milk enzyme. It has been used as reporter for process controls in food industry. Since ALP denatures at pasteurization temperature (at 63°C or 72°) its detection in milk confirms the unproper pasteurization. There are different detection procedures such as colorimetric, fluorometric methods and immunoassays for ALP in milk. However, they are time consuming processes and require specific instruments and qualified staff. In this study, new, semiquantitative, disposable, cheap and practical paper bioreporter have been developed for ALP detection.

In optimization studies, 1mg/mL p-NPP in 0.1 M glycine buffer at pH 9.5 and 0.5 mg/mL bromocresol green in 1.0 M Tris-HCl buffer at pH 9.5 were determined as optimum for ALP bioreporter as a result of visual inspection and green color intensity analyses.

The effects of samples temperature and pH of on the response of bioreporter were tested. Milk samples at pH 5.0, 5.5, 6.0 and 6.5 and milks stored at 37°C, room temperature and 4°C did not affect the response of bioreporter.

Also the response of bioreporter against milk samples from different animals (cattle, sheep and goat) and cow's milk from different location in Turkey were evaluated. The appropriate responses were observed by bioreporter.

Whatman filter papers, cotton and bandage were used as support materials to construct bioreporter and Whatman filter papers were selected as the most applicaple support material.

Finally, stability tests were carried out at 4°C and room temperature and 40 days at 4°C was determined as shelf life of bioreporter.

Key words: Alkaline phosphatase, Bioreporter, Milk, Pasteurization

ÖZ

SÜT PASTÖRİZASYONUNUN BELİRLENMESİ İÇİN ALKALEN FOSFATAZ TABANLI KAĞIT TİPİ BİYORAPORTÖRÜNÜN GELİŞTİRİLMESİ

Karakaş, Ceren
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi : Prof. Dr. Hüseyin Avni Öktem
Ortak Tez Yöneticisi : Dr.Can Özen

Haziran 2009, 148 sayfa

Alkalen fosfataz çiğ sütte bulunan doğal bir enzimdir. Bu enzim gıda sektöründe işlem kontrolleri için muhabir olarak kullanılabilir. Pastörizasyon işlemi sırasında (63°C yada 72°C) denatüre olduğu için sütün içerisindeki tanısı yapılan pastörizasyon işleminin doğru yapılmadığının göstergesi olarak kullanılır. Günümüzde sütteki alkalen fosfatazın tespiti için spektrofotometrik, kalorimetrik ve florometrik yöntemler vardır. Fakat bu yöntemler zaman alan işlemlerdir ve özel araç ve deneyimli personel gerektirir. Bu çalışmada, alkalin fosfatazın tanısı için yeni,yarı kantitatif tek kullanımlık, ucuz ve pratik kağıt tipi biyoraportör geliştirilmektedir.

Optimizasyon çalışmasında, görsel verilerin ve renk analizinin sonuçlarına göre 1 mg/mL p-NPP 0.1 M glisin tampon çözeltisinde ve 0.5 mg/mL bromokresol mavisi 1.0 M Tris-HCl tampon çözeltisinde ALP tanısı için optimum olarak saptanmıştır.

Süt örneklerinin pH ve sıcaklıklarının biyoraportör cevabı üzerindeki etkisi test edilmiştir. pH 5.0, 5.5, 6.0 ve 6.5 ta olan süt örnekleri ve 37°C, oda sıcaklığı ve 4°C de bekletilen süt örneklerinin biyoraportörün cevabını etkilemediği görülmüştür.

Ayrıca farklı hayvanlardan (sığır, koyun ve keçi) ve Türkiye'nin farklı bölgelerinden gelen inek sütü örneklerinin biyoraportörün cevabı üzerindeki etkisi değerlendirilmiştir. Biyoraportör tarafından uygun cevap alınmıştır.

Whatman filtre kağıdı, pamuk ve sargı bezi biyoraportötün yapımı için destek materyali olarak kullanılmış ve en uygun materyali olarak Whatman filtre kağıdı seçilmiştir.

Son olarak biyoraportörün kararlıklık çalışmaları 4 santigrat derecede ve oda sıcaklığında gerçekleştirilmiş ve biyoraportörün raf ömrü 4 santigrat derecede 40 gün olarak belirlenmiştir.

Anahtar kelimeler: Alkalen fosfataz, Biyoraportör, Süt, Pastörizasyon

To My Family

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Prof. Dr. Hüseyin Avni Öktem for his guidance, encouragements,valuable comments and endless supports. I would also like to express my gratitude to Prof. Dr. Meral Yücel for her encouragement, valuable supports and precious remarks. I am pride of being a member of their laboratory.

I would like to thank my co-supervisor Dr. Can Özen for insight, helps, valuable suggestions and comments during the study.

I would like to thank to all my thesis committee for their suggestions and criticism.

I am also thankful to Prof. Dr. Azmi Telefoncu for valuable suggesstions and criticisms during this study.

I would like to thank Atatürk Orman Çifliği, Milk Products Factory and Veterinary Department of Ankara University for the support of raw milk samples from different location in Turkey and from different animal sources, respectively.

I want to thank Dr.Ufuk Çelikkol Akçay, Hamdi Kamçı, Beray Gençsoy, Musa Kavas, Gülsüm Kalemtaş, Cengiz Baloğlu, M. Tufan Öz, Taner Tuncer, Oya Ercan, Ceyhun Kayıhan, Emre Aksoy, Nilufer Afşar, A.Hamit Battal, Ayten Eroğlu, Özge Şenyurt, , Murat Kavruk, B. Batuhan Keskin and Berrak Yanıkömeroğlu one by one for their splendid technical and moral supports, valuable comments and sincere friendships.

I really appreciate Sena Cansız, Fatma Kılbacak, A. Çınar Adalı and Lutfiye Yıldız for their endless love, patiences, encouragments and and being one of important part of my life. I believe you are with me throughout my life.

My special thanks and gratitude go to A. Tahir Bayraç, for being precious part of my life, for his endless love and supports. I am thankful to him for encouragement, endless patience, valuable suggestions and scientific guidances in the study.

I owe my sincere gratitude to my family, Ali Karakaş, Sibel Karakaş, Volkan Karakaş, Hilal Baran Karakaş , İlker Karakaş and my niece Irmak Karakaş for their endless love ,thrust , encouragement and support at all stages of my life.

It is my duty to express my gratitude to TUBITAK for the support that I have received as scholarships during my graduate education.

This study was granded by Nanobiz Nano-BioTechnological Systems Education-IT-Consultancy R&D Limited (TUBITAK-TEYDEP-7080562).

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

LIST OF ABBREVATION

p-NPP	p-nitrophenly phosphate
ALP	Alkaline phosphatase
UHT	Ultra-high temperature

CHAPTER 1

INTRODUCTION

1.1 Biosensors

The idea of measuring some analytes in blood by an oxygen electrode had arised the concept of biosensor in 1962 (Clark & Lyons, 1962). Professor Leland C Clark Jnr was inventor of the first sensor measuring the glucose concentration in blood with the use of an enzyme, glucose oxidase, and oxygen electrode (Clark & Lyons, 1962). Since the invention of the first electrochemical sensor for glucose detection, the research area throughout the world has been focused on the development of many biosensors for a variety of analytes. The definition of biosensor has been stated at different studies in literatures; however, the main idea has been agreed on the components of biosensor.

According to Mello and Kubota (2002), " Biosensors are a sub group of chemical sensors in which are analytical devices composed of a biological recognition element (such as enzyme, antibody, receptor or micro- organisms) coupled to a chemical or physical transducer (electrochemical, mass, optical and thermal)".

As shown in Figure 1.1, bioreceptor with the binding of an analyte converts specific response to a quantitative signal by a transducer coupled to it (Luong *et al.*, 1997). The quantitative signal represented by biosensor is correlated with the concentration of analyte (Sharma *et al.*, 2003).

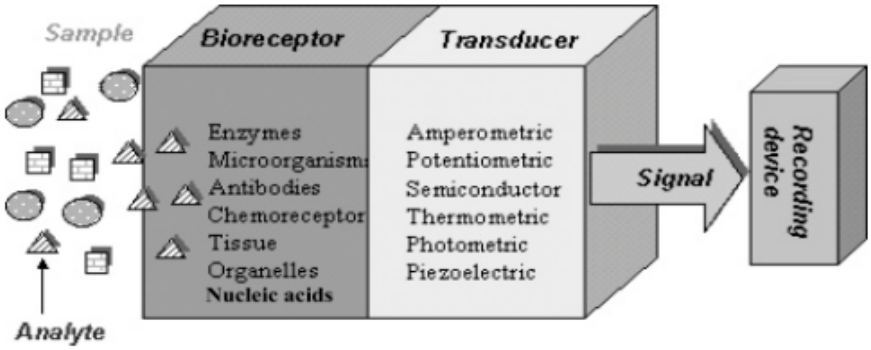

Figure 1.1.The representative figure of biosensor and its components (Newman *et al*. 2004; Terry *et al.,* 2005)

The increase in the demands for practical and reliable methods for the detection of different analytes has been lead to the development of biosensors. Their advantages over classical techniques such as spectrophotometry and chromatography have made them significant for a wide range of area (Mello & Kubota, 2002). The biosensors have shown high selectivity and specificity for the analytes to be detected owing to their biological recognition element (Chaplin, 2000). Generally, they are tolerable to the changes in the condition such as temperature and pH. The main advantages of biosensor which make them the most effective system today are their low production cost, portability and the opportunity for fast analysis (Chaplin, 2000; Mello & Kubota, 2002).

1.2 Classification of Biosensors

The increase in the application area therefore the increase in the types of analyte has given rise to the variety in the biological recognizer and transducer for the construction of biosensor. These two components of biosensor have been changed according to the specific properties of analytes in sample and the types of signal to be measured. In the consequences of these varieties the classification of

biosensors can be performed according to the biological element and the mode of signal to be transferred (Mello & Kubota, 2002). The Figure 1.2 (Mello & Kubota, 2002) represents some analytes, recognizer components for the detection of these analytes and different transducers for the conversion of responses to specific signals.

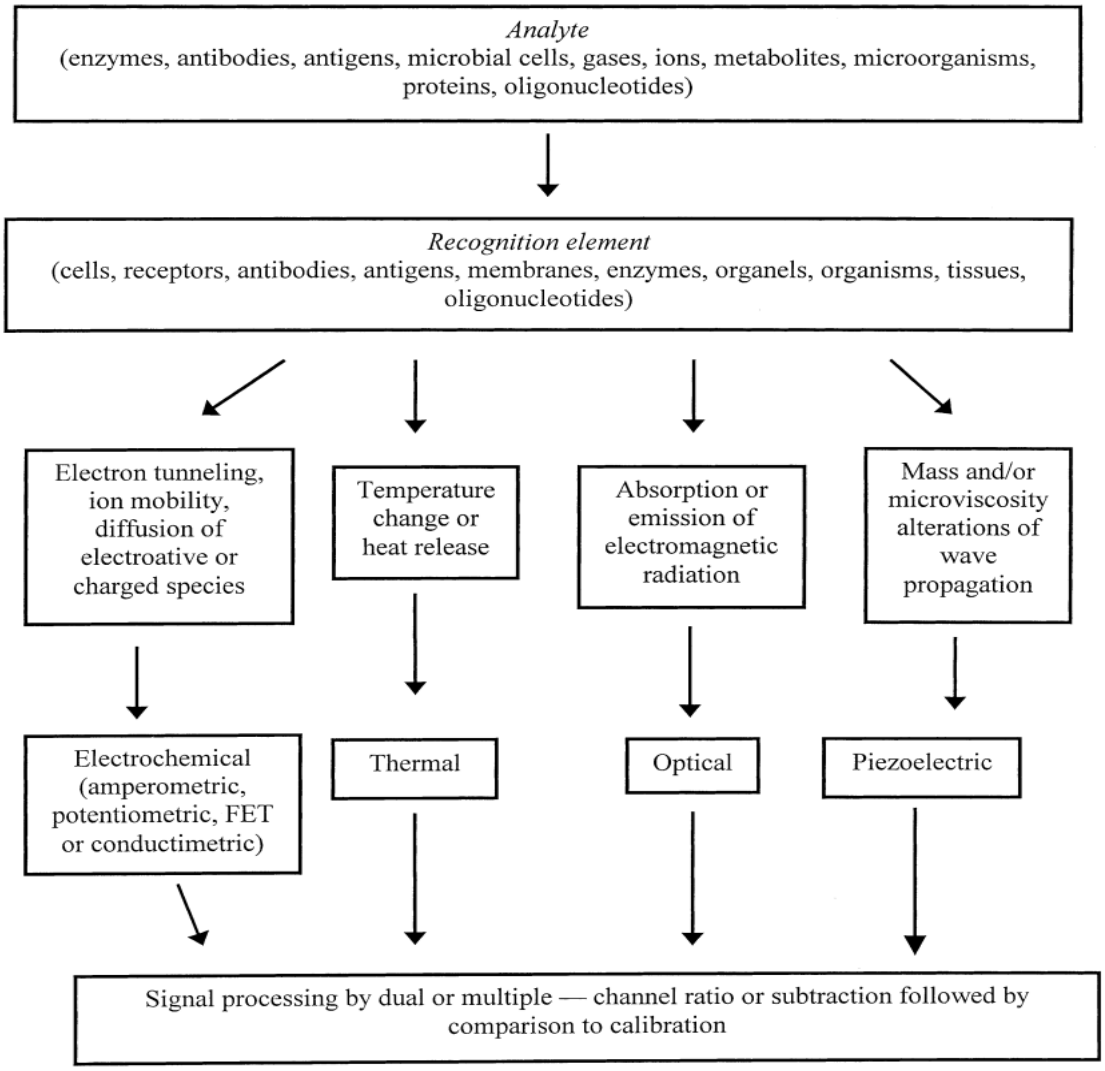

Figure1.2. Biocomponent and transducers employed in construction of biosensors (Mello & Kubota, 2002)

1.2.1 Classification of Biosensors According to Bioreceptor Element

As it is mentioned, the biological elements have provided biosensors with high selectivity and specificity for the analytes (Mello & Kubota, 2002). The biological elements, enzymes, antibodies, antigens, cells, organelles, proteins, aptamers, oligonucleotides, can be used in the construction of biosensor and their recognition properties have given rise to two basic groups of biosensor which are the biocatalytic and bioaffinity biosensors (Mello & Kubota, 2002).

1.2.1.1 Biocatalytic biosensor

In this type of biosensor, binding of biological elements with the analytes is based on the biocatalytic reactions (D'Orazio, 2003). The measurements of rate of product formation, the consumption of substrate and inhibition of the reaction have been correlated with the concentration of analytes in samples (Marazuela & Moreno-Bondi, 2002). The typical recognition element can be enzyme, whole cells from different microorganism and even the piece of tissue from animal or plant (Davis *et al*., 1995). Among these the usage of enzyme as biological element is common for the construction of biosensor (Thévenot *et al*., 2001, Marazuela & Moreno-Bondi, 2002). The opportunity of having different transduction principles has made enzyme as the commonly used biological elements in biosensor construction (Marazuela & Moreno-Bondi, 2002).

As it is mentioned, the first biosensor described by Clark and Lyons was for the detection of glucose in blood.

$$\text{glucose} + O_2 \longrightarrow \text{gluconic acid} + H_2O \qquad (1)$$

The basic principle was the measurement of the consumption of oxygen in the oxidation reaction of glucose with glucose oxidase immobilized to surface of

biosensor (Clark & Lyons, 1962; D'Orazio, 2003). After this study, different enzymes were used for the construction of biosensor for the application in different area.

For the determination of penicillin production in fermentation medium, the enzyme penicillinease was immobilized on an electrode surface. The immobilized penicillinase converted penicillin into penicilloic acid; the resulted pH changes in medium represented the presence of penicillin concentration (Guilbault, 1984).

Another example, the aspartame biosensor (Guilbault *et al*., 1988) was constructed by the immobilization of carboxypeptidase and L-aspartase on electrode. The aspartame was cleaved by the action of carboxypeptidase and the intermediate product, L-aspartic acid was deaminated by L-aspartase. The ammonium ions as an end product were detected with electrodes.

In addition to enzymes whole cells have been used as biological elements for the recognition of analytes. The usage of whole cells has provided many advantages (Marazuela & Moreno-Bondi, 2002). The changes in pH and temperature of medium have not shown significant effect on the responses of whole cell based biosensors as compared with enzyme based biosensors. Instead of isolation and purification of enzyme some cells such as bacteria and yeast have been easily isolated from natural sources and applied to biosensor. Moreover, cells have provided many different enzymes to detect different analytes without any cofactor requirement from outside (Marazuela & Moreno-Bondi, 2002).

In literature, different studies have shown the use of bacteria, yeast, plant and animal cells in the construction of whole cell based biosensor. In the Table 1.1 (Mello & Kubota, 2002), some microbial biosensors have been summaried.

The animal and plant tissue slices have been also studied as recognizing element in biosensors. For example, biosensor constructed with algae cells has detected the presence of herbicides in wastewater by measuring the chlorophyll fluorescence (Frense *et al.,* 1998)

Table 1.1 Microorganisms used as biomolecules for development of microbial biosensors and their applications (Mello & Kubota, 2002)

Biomaterials (microorganisms)	Type of sensor	Applications
Azotobacter vinelandii	Nitrate	Ammonia sensor in industrial process
B. subtilis	Mutagenes	Screening of mutagens by microbial sensor
Bacterium cadaveris	Aspartic acid	Amino acid sensor for healthcare and food industry
Citrobacter fseundi	Formic acid	Fermentation industry, culture media, urine, blood and gastric juices
Clostridium acidurici	L-serine	Amino acid sensor for healthcare indicators
Desulfovibrio desulfuricans	Sulfate	Agriculture industry
E. coli	L-tryptophan	Amino acid sensor
E. coli	Lysine	Amino acid sensor for fermentation and food industry
E. coli	Glutamic acid	BOD, healthcare and food industry
E. coli	Glutamic acid	BOD, healthcare and fermentation
Enterobacter agglomerans	Ascorbic acid	Pharmaceutical industry
L. arabinosis	Nicotinic acid	Drug industry
Methylomonas flagelatis	Methane	Fermentation industry
Nitrifyingbacteria (*Nitrosomonas* and *Nitrobacter* sp.)	Ammonia	Clinical and industrial process
N. erythropolis	Cholesterol	Blood cholesterol and food industry
Proteus morganii	L-cysteine	Amino acid sensor
P. fluorescens	Glucose	Molasses industry
Pseudomonas sp.	L-histidine	BOD in waste water
S. cerevisiae	Glucose, sucrose, fructose	Food industry and beverages
Sarcina flava	Glutamine	Amino acid sensor
Streptococcus faecium	L-arginine	Amino acid sensor
T. brassicae	Acetic acid	Fermentation industry
T. brassicae	Ethanol	Fermentation industry
T. cutaneum	BOD	Environmental pollution monitor

1.2.1.2 Bioaffinity biosensor

The basic principle of bioaffinity biosensors is different from the catalytic reaction between the biological element and analytes as in the biocatalytic biosensor. The presence of higher affinity leads to the binding reaction between biological elements and analytes and the stable complex formed induces the physicho-chemical change. The bioaffinity biosensors, therefore, relies on this change for the detection of analytes (Mello & Kubota, 2002).

The biological elements in the construction of bioaffinity biosensor are antibodies, receptors and nucleic acids (Mello & Kubota, 2002; Sharma *et al*., 2003). The most common bioaffinity biosensor is the immunosensor which is based on the specific binding of antibody with antigen. By labeling antibody or antigen with certain enzymes such as peroxidase, alkaline phosphatase and glucose oxidase or with fluorescent compounds, electrochemically active substrates, radionuclides or avidin–biotin complexes the analytes are sensed as a result of detected signals (Scheller *et al.*, 2001; Mello & Kubota, 2002).

1.2.2 The Classification of Biosensors According to Transducer Element

The detection of analyte by biosensor is monitored in different ways according to the interaction between the biological elements and the analytes. The consumption rate of oxygen, the production of hydrogen peroxide, the changes in pH, temperature, conductivity and mass have provided biosensor with the variety in the type of transducer. As shown in Figure 1.2 (Mello & Kubota, 2002), biosensors are classified according to their transducer as electrochemical, optic, piezoelectronic and thermal biosensors (Mello & Kubota, 2002). The advantages and application area of transducer are summarized in Table 1.2 (Mello & Kubota, 2002).

Table 1.2 Types of transducers, their characteristics and application (Mello & Kubota, 2002)

Transducer	*Advantages*	*Disadvantages*	*Application*
Ion-selective electrode (ISE)	Simple, reliable, easy to transport.	Sluggish response, requires a stable reference electrode, susceptible to electronic noise.	Amino acids, carbohydrates, alcohols and inorganic ions
Amperometric	Simple, extensive variety of redox reaction for construction of the biosensors, facility for miniaturize.	Low sensitivity, multiple membranes or enzyme can be necessary for selectivity and adequate sensitivity.	Glucose, galactose, lactate, sucrose, aspartame, acetic acid, glycerides, biological oxygen demand, cadaverine, histamine, etc.
FET	Low cost, mass production, stable output, requires very small amount of biological material, monitors several analytes simultaneously.	Temperature sensitive, fabrication of different layer on the gate has not been perfected.	Carbohydrates, carboxylic acids, alcohols and herbicide
Optical	Remote sensing, low cost, miniaturizable, multiple modes: absorbance, reflectance, fluorescence, extensive electromagnetic range can be used.	Interference from ambient light, requires high-energy sources, only applicable to a narrow concentration range, miniaturization can affect the magnitude of the signal.	Carbohydrates, alcohols, pesticide, monitoring process, bacteria and others…
Thermal	Versatility, free from optical interferences such as color and turbidity.	No selectivity with the exception of when used in arrangement	Carbohydrates, sucrose, alcohols, lipids, amines
Piezoelectric	Fast response, simple, stable output, low cost of readout device, no special sample handling, good for gas analysis, possible to arrays sensors.	Low sensitivity in liquid, interference due to non specific binding.	Carbohydrates, vitamins, pathogenic microorganisms (e.g. *E. coli*, Salmonella, Listeria, Enterobacter), contaminants (e.g. antibiotics, fungicides, pesticides), toxic recognition as bacterial toxins.

1.2.2.1 Electrochemical Biosensors

"The basic principle of electrochemical sensors is that the electroactive analyte species is oxidized or reduced on the working electrode surface, which is subjected to some predefined pattern of fixed or varying potential, and the change in

electrical parameters resulting from redox reaction, as a function of the type or concentration of analyte, is measured" (Ahmed *et al*., 2008). The electrochemical biosensors provide many areas with the advantages of low cost for production and fast analysis for different analytes (Luong *et al.*, 1988). The electrochemical biosensors are furthered classified into conductometric, potentiometric and amperometric biosensors (Mello & Kubota, 2002).

1.2.2.1.1 Conductometric Biosensor

In conductometric biosensors the detection of analytes relies on the changes in conductivity of medium by the action of recognizing element (Mello & Kubota, 2002). Generally, enzymes are used as biological element to detect the changes in ionic strength, therefore the conductivity, resulted from the enzymatic reaction between anayte and immobilized enzyme (Grieshaber *et al*., 2008). The detection of drug in human urine is one of the examples for the application of enzyme based conductometric biosensors for the diagnostic purposes (Yagiuda *et al*., 1996). In addition to enzymes, cells are also studied as recognizer in the construction of conductometric biosensor (Chouteau *et al.,* 2004). Although there are different studies for conductometric biosensor, it is not generally preferred in use due to its drawbacks, such as being non specific and having poor signal/noise ratio (Mello & Kubota, 2002).

1.2.2.1.2 Potentiometric Biosensor

The change in the ion concentration or pH of the medium forms the basis of the working principle of potentiometric biosensor (Mello & Kubota, 2002). Three different electrodes, ion- selective electrodes (IES), coated wire electrodes (CWES) and field effect transistors (FETS) can be used to measure the potential differences between working and reference electrodes (Stradiotto *et al.,* 2003). As recognizing

elements enzymes, antigens and antibodies are immobilized on transducer and changes in the potential due to enzymatic reactions or complex formation between antigens and antibodies are measured in this type of biosensor (Mello & Kubota, 2002). The most common example of potentiometric biosensor is the pH biosensor (Stradiotto *et al.,* 2003).

1.2.2.1.3 Amperometric Biosensor

"The amperometric biosensors measure the current produced for the chemical reaction of an electroative species to an applied potential, which is related to the concentration of the species in solution" (Mello & Kubota, 2002). The basic principle is the transfer of electrons from or to analytes as a result of oxidation-reduction reaction (Mello & Kubota, 2002; Prodromidis & Karayannis, 2002).

The detection of analytes in medium is correlated with the consumption of oxygen, the formation of hydrogen peroxide(H_2O_2) or the reductation of β-nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) in the reaction medium (Prodromidis & Karayannis, 2002).

The most famous amperometric biosensor is the glucose biosensor which was first constructed by Clark and Lord in 1962. The immobilized enzyme, glucose oxidase, degrades glucose molecules with the consumption of oxygen and the measurement of oxygen level amperometrically was correlated with the concentration of glucose in medium (Clark & Lyons, 1962; D'Orazio, 2003; Mello & Kubota, 2002).

Another example of amperometric biosensor is the ATP biosensor (Kueng *et al.,* 2004). The two enzymes, glucose oxidase (GOD) and hexokinase (HEX) were immobilized to the surface of electrode and the degradation of glucose by the

action of these enzymes leaded to decrease in signal compared to ATP concentration.

1.2.2.2 Optic Biosensors

"An optical fiber-based biosensor is a biosensor that employs an optical fiber or optical fiber bundle, as a platform for the biological recognition element, and as a conduit for excitation light and/or the resultant signal" (Monk & Walt, 2004). They measure the responses to illumination or to emission of light resulting from the interaction between the analytes and recognizer element (Mehrab et al., 2000). The detection principles of optic biosensors are provided from UV–Vis absorption, bio/chemiluminescence, fluorescence/phosphorescence, reflectance, scattering and refractive index (Mello & Kubota, 2002). According to the recognition elements used for construction optical fiber based biosensors are classified into five groups; enzyme, immunoassay, nucleic acid, whole cell and biomimetric optical fiber-based biosensors.

In the enzyme optical fiber based biosensor, the reaction between immobilized enzymes with its substrate gives product with optical properties such as absorbance, reflectance or fluorescence (Monk & Walt, 2004). As an example, the detection of pesticides with the use of acetylcholinesterase was studied by Andreou and Clonis (Andreou & Clonis, 2002). The change in pH as a result of enzymatic activity was monitored with the pH absorbance indicator bromocresol purple (Andreou & Clonis, 2002).

The immunoassay optical fiber based biosensor is based on the detection of an optical properties resulted from the interaction between antibody and antigen (Monk & Walt, 2004). The biotin-labeled primary antibody and Cy 5 dye labeled

secondary antibody were used for the detection of protein C (PC) can be shown as an example for immunoassay optical fiber based biosensor (Balcer *et al*., 2002).

The hybridization of single stranded DNA with its complementary sequence forms the of detection principle in nucleic acid optical fiber based biosensor. One of the single stranded DNA is immobilized to optical surface and hybridized with other sequence which is labeled with optical indicator such as fluorescens (Monk & Walt, 2004).

The whole cell optical fiber based biosensor uses the cell for the detection of optical property resulting from the interaction between analytes and cells. Generally this type of biosensor is studied for the detection of toxic molecules with genetically engineered bioluminescent bacteria (Monk & Walt, 2004; Gu *et al*., 1999; Gu & Gil ,2001) In biomimetric optical fiber-based biosensors the detection of analytes is performed by a nonbiological element which resembles to a biological recognizer based on its selectivity (Monk & Walt, 2004).

Optical biosensors provide certain advantages such as being accurate and providing better signal/noise ratio even though the construction processes are costly (Monk & Walt, 2004).

1.2.2.3 Piezoelectric Biosensors

In piezoelectronic biosensor, " analyte detection is based on adsorbate recognition where selective binding causes a mass change which is identified by a corresponding change in the acoustic parameters of the PQC **"** (Bunde *et al.,* 1998). The change in the frequency of oscillation as a consequence of change in the mass of crystal is correlated with the interaction between the analytes and recognition elements (Mello & Kubota, 2002). Different studies were performed to

construct piezoelectronic biosensors for the detection of pathogenic microorganisms, gases, aromas, pesticides, hormones and others (Mello & Kubota, 2002; Abad *et al*., 1998; Horacek *et al.,* 1998; Bizet *et al*., 1999; Ivnitski *et al.,* 1999; Wu, 1999; Babacan *et al.,* 2000).

1.2.2.4 Thermal Biosensors

Thermometric biosensors measure the change in energy resulting from the production or absorption of heat during a reaction between analytes and biological recognizers (Ramanathan & Danielsson, 2001; Mello & Kubota, 2002). The enzyme, microbial cells and antibodies are used in thermal biosensor as recognizing element, however, the application of enzymes are common due to their high selectivity and stability (Ramanathan *et al.,* 1999; Mello & Kubota, 2002). Thermal biosensors are used for the detection of different analytes such as ethanol (Guilbault *et al*., 1983), glucose (Mandenius *et al.,* 1985), oxalate (Winquist et al., 1985), urea (Xie *et al.,* 1994), ascorbate (Mattiasson & Danielsson, 1982), penicillin (Decristoforo & Danielsson, 1984), heavy metal ions such as Hg2+, Cu+2 and Ag+ (Mattiasson *et al*., 1978; Preininger & Danielsson, 1996) and retinol (vitamin A) (Ramanathan *et al.,* 2000).

1.3 Application of Biosensors in Food Industry

The development in the production of biosensor for a variety of analytes has shown a significant effect in food industry. The applications of biosensor have replaced the use of analytical methods which are time consuming and uneconomical. In food industry, biosensors are used to determine the composition of product, to ensure the safety of food and to control the process (Mello & Kubota, 2002).

In literature there are different studies for the construction of biosensors for the monitoring of carbohydrates, alcohols, amino acids, amines, organic and inorganic compounds and contaminants such as microorganisms, toxins, pesticides and additives. Examples of each compound have been given in the following part.

The determination of fructose content in honey was performed by the action of D-fructose dehydrogenase immobilized on the surface of electrode (Bassi *et al.,* 1998).The fructose content of juice and sweetener was also shown by amperometric biosensors (Boujtita *et al*., 2000; Garcia *et al*., 1998). The lactose content of milk was determined potentiometrically with the biological elements, β-galactosidase, lactozym and *Saccharomyces cerevisia* (Ama´rita *et al*., 1997).

Alcoholic beverages such as wine and beer were tested for their ethanol content with the use of enzyme based biosensors. In these biosensors alcohol dehydrogenase and alcohol oxidase were immobilized to amperometric electrode surface and the changes in the current were correlated with the alcohol content in certain beverages (Boujtita *et al.,* 2000; Katrlı´k *et al.,* 1998).

In order to determine the L-amino acid content of milk, fruit juice and wine different amperometric biosensors were constructed. L-glutamate oxidase, L-lysine-α-oxidase and L-malate dehydrogenase were used as recognizer elements to detect the presence of glutamate, lysine and malate in beverages respectively (Matsumoto *et al.,* 1998; Curulli *et al.,* 1998; Gajovic *et al.,* 1997).

The levels of accumulation of xanthine and hypoxanthine in fish are the markers for fish freshness; therefore, biosensors constructed with xanthine oxidase were used to control the freshness of fish (Mao & Yamamoto, 2000).

The contamination of foods with bacteria, especially with *E.coli* and *Salmonella* was determined by biosensors with different transducers. Instead of enzymes, antibodies were used for the detection of bacteria in food, chickens and egg (Hamid *et al.,* 1999; Su *et al*., 2001).

In literature, the detection of pesticides, herbicides and toxins in foods, vegetables and fruits were also studied. For the construction of these biosensors, certain enzymes (acetylcholinesterase, tyrosinase and choline oxidase) and antibodies (Anti-aflatoxin antibody, Anti-Staphylococcal enterotoxin B antibody) were immobilized to the surface of amperometric, optic, potentiometric and piezoelectronic transducers (Medyantseva *et al.,* 1998; Pita *et al.,* 1997; Palchetti *et al*., 1997; Carter *et al*., 1997; Rasooly & Rasooly, 1999).

In addition to the determination of composition biosensors are used to control the processes in food industry. The fermentations, biotransformations, downstream processes are controlled with use of biosensors (Becker *et al*., 2007).

Biosensors can also be used to control different processes in food industry for the improvements of the effectiveness of processes (Ferreira *et al*., 2003). The use of biosensors is generally for the control of pH, temperature of reaction medium, concentration of oxygen, carbon dioxide, substrate and product concentrations in fermentation medium (Ferreira *et al*., 2003). As an example, the control of glucose concentration during the fed-batch fermentation process by the use of glucose biosensor was studied by Hitzmann *et al* (Hitzmann *et al*., 2000). The lactose biosensor was also studied to control the production of β-galactosidase by the yeast *Kluyveromyces marxianus* in fermentation medium (Ferreira *et al*., 2003).

1.4 Pasteurization of Milk

Pasteurization which is one of the thermal processes applied in food production plays important roles in food industry**.** The application of pasteurization process provides with milk and milk products to have longer shelf life and to ensure the safety of end product (Dinnella *et al.,* 2004).

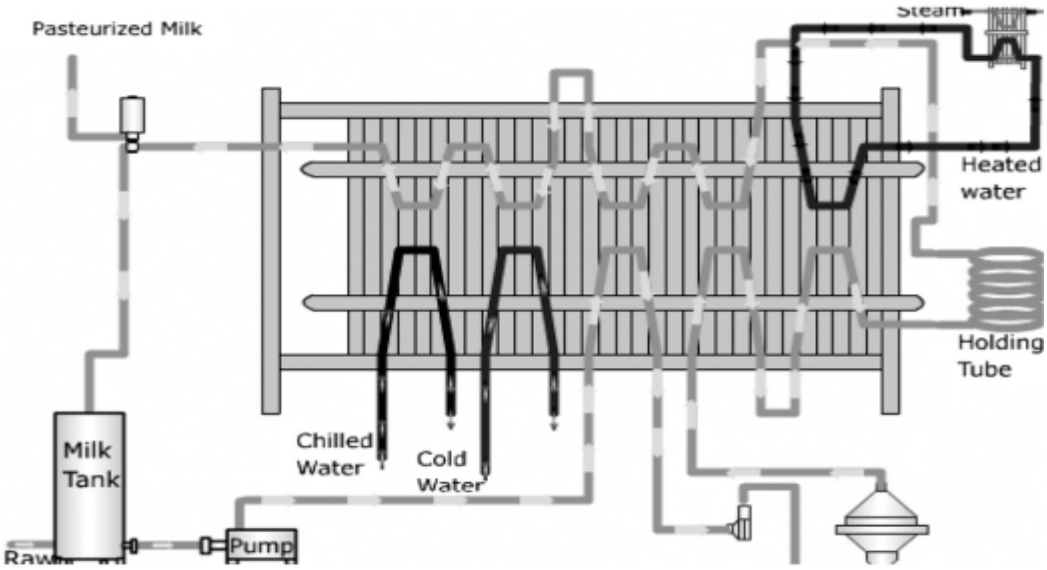

Figure 1.3 The representative figure of pasteurizer of milk (Riverol et al., 2008)

At the first step of the pasteurization process, raw milk is heated to pasteurization temperature (63˚ or 72˚) while passing through plate heat exchanger as shown in Figure 1.3 (Riverol et al., 2008). At holding tube heated milk is stored for 30 min at 63˚C or 15 sec at 72˚C to maintain holding time. Then, pasteurized milk is chilled at cooling part of the system (Riverol et al., 2008).

Since the pasteurization process is the last and the important step for the milk production before reaching to consumers, pasteurized milk has to be controlled to ensure the desired properties (Mortier *et al*., 2000). Pasteurization of milk has to eliminate pathogenic microorganisms from the end product and increase the shelf life of the product without damaging the nutritional value of milk (Mortier *et al*., 2000).

In literature, there are three different approaches for the control of thermal processes in food industry (Van Loey *et al.,* 1996; Dinnella *et al*., 2004). In the first approach, the effectiveness of process is determined by comparing the level of microorganisms in product before and after the application of heat. The second approach depends on the characteristic of microorganism upon the application of heat treatment. The effects of heat treatment and its duration are evaluated quantitatively as the number of microorganisms in product. The last approach makes the use of indicator to control the efficiency of process (Dinnella *et al*., 2004). The indicator used to evaluate the effectiveness of time-temperature combination in thermal process is stated by Sherlock *et al*. as ‘‘an indicator is a small device that shows for a selected temperature, easily, accurately and precisely measurable irreversible changes that mimic the change of a target attribute undergoing the same temperature exposure’’ (Sherlock *et al*., 1991). The best molecules providing the requirements as indicator are the enzymes. The conformational changes leading to the denaturation of enzymes as a result of heat treatment are easily evaluated (Dinnella *et al.,* 2004). The enzymes as the indicator for the effectiveness of thermal process are generally endogenous enzymes (Mortier *et al*., 2000; Dinnella *et al*., 2004).

1.5 Alkaline phosphatase as An Indicator in Pasteurization Process

Alkaline phosphatase (ALP) is a membrane-bound protein found naturally in unpasteurized milk including bovine milk (Vega-Warner et al. 1999). Bovine ALP (EC 3.1.3.1; phosphatase) is a glycoprotein containing sialic acid with molecular weight of 187 kDa (Chen *et al.,* 2006). Bovine ALP contains at least three isoforms which typically possess two N-linked and one or more O-linked glycans per monomer (Neumann & Lustig, 1980). The enzymatic reaction of alkaline phosphatase is optimum at pH 8 to 10 however the optimum pH value depends on the substrate used in enzymatic reaction.

The thermal inactivation of alkaline phosphatase in milk is performed at a slightly higher time-temperature combination than sterilization condition of pathogenic microorganisms especially *Mycobacterium tuberculosis*, therefore, the assay for the detection of alkaline phosphatase in milk after pasteurization is used as an indicator of the effectiveness of process (Griffiths, 1986; Andrews *et al.,* 1987; McKellar & Emmons, 1991; Wilin´ska *et al.*, 2007). The heat treatment at 63˚C for 30 min or 72˚C for 15 sec which represent the time-temperature combination of pasteurization process inactivates ALP as well as pathogenic microorganisms in milk (Vega-Warner et al., 1999). Due to its higher thermal resistance compared with that of microorganisms ALP is used widely for the indication of lower limit of pasteurization process (Fadıloğlu *et al*., 2006; Wilin´ska *et al.*, 2007). The detection of alkaline phosphatase was performed with the use of different substrate selected according to the detection principles. For example, based on colorimetric detection methods p-nitrophenyl phosphate and phenolphthalein phosphate were selected as substrate (Ishikawa, 1987; Wilkinson & Vodden, 1966; Zhu et al., 2006). The electrochemical detection of ALP was studied with p-cyanophenyl phosphate, 1-naphthyl phosphate, 4-hydroxy-naphthyl-1-phosphate, 4-amino-1-naphthyl

phosphate, (Kreuzer *et al.,* 1999; Gehring, *et al*., 1999; Masson *et al*., 1999; Masson et al., 2004; Zhu et al., 2006). As chemiluminescent substrate, cortisol-21-phosphate, adenosine-3'-phosphate-5'-phosphosulfate were used in different assay systems (Lin *et al*., 1997; Kokado *et al*., 1997; Arakawa *et al*., 2003; Zhu *et al*., 2006). Salicylic acid phosphate, 4-methylumbelliferone phosphate were used for fluorescent assay (Lianidou *et al.,* 1994; Zhu *et al.,* 2006).

In literature different methods were studied for the determination of alkaline phosphatase in dairy products.

Fenoll *et al*. (2002) studied the determination of alkaline phosphatase in fluid dairy products with the use of fluorimetric method. The basic principle depended on the formation of highly fluorescence product from the cleavage of non-fluorescent aromatic monophosphoric ester substrate by the action of ALP.

Geneix *et al*. (2007) produced monoclonal antibody specific for bovine ALP and performed immunoassay for the detection of bovine alkaline phosphatase with p-NPP substrate. Their aim was to determine the bovine alkaline phosphatase activity in milk without the effect of microbial source of ALP.

The composite amperometric tyrosinase biosensor was developed for the detection of alkaline phospatase by Serra *et al.* (2005). The princible of tyrosinase biosensor was the detection of phenol generated by the action of ALP at the tyrosinase composite electrode through the electrochemical reduction of the o-quinone produced to catechol.

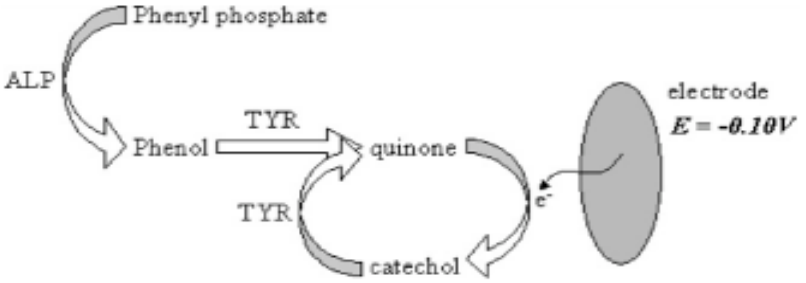

Figure 1.4.Schematic representation of alkaline phosphatase detection at the graphite–Teflon–tyrosinase electrode (Serra et al., 2005)

Sharma *et al*. (2003) developed a strip to detect ALP in milk samples for the testing of effectiveness of pasteurization process. They immobilized substrate and chromogen to support material and the detection principle was based on the color change on paper.

Chen *et al*. (2006) used the immunoglobulin in yolk (IgY) specific against bovine milk alkaline phosphatase as the detection element in competitive indirect enzyme-linked immunosorbent assay (CI-ELISA). They produced primary antibody specific for bovine alkaline phosphatase and used the antibody in assay of enzyme activity.

Vega-Warner *et al.* (2000) provided a detection method for milk ALP with the use of polyclonal antibody in enzyme-linked immunosorbent assay (ELISA). Their aim was to develop ELISA method with poly-clonal antibody against milk alkaline phosphatase for the quantification of ALP in milk as an indication of adequate pasteurization.

1.6 Aim of the study

In this study, the aim is the development of a paper bioreporter for the determination of alkaline phosphatase in milk as a sign of pasteurization efficiency. In the literature, there are different studies for the determination of residual alkaline phosphatase activity in pasteurized milk such as spectrophotometric, fluorometric methods and immunoassay. However, these methods are time consuming and expensive. They also requires qualified staff and specific instruments. As a result, the construction of this bioreporter has been completed the needs for cheap, practical, semiquantitative and single use bioreporter for validation of pasteurization process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Alkaline phosphatase was purchased from Applichem Company. 4-nitrophenyl phosphate disodium salt hexahydrates (p-NPP disodium salt hexahydrates) and bromocresol green were purchased from Fluka and Sigma Chemical Company respectively.

2.1.2 Support Materials

As support material Whatman filter paper No .1, cotton of ear cleaning rod and bandage were used. Whatman filter paper No .1 was purchased from Whatman.

2.1.3 Milk Samples

Different milk samples were taken from milk and dairy products factory of Atatürk Orman Çifliği and Veterinary Faculty of Ankara University in ANKARA. The commercial UHT milk (Pınar Süt) was used for the preparation of milk samples containing different amount of alkaline phosphatase and also as a control.

2.2 Methods

2.2.1 Construction of Paper Based Bioreporter

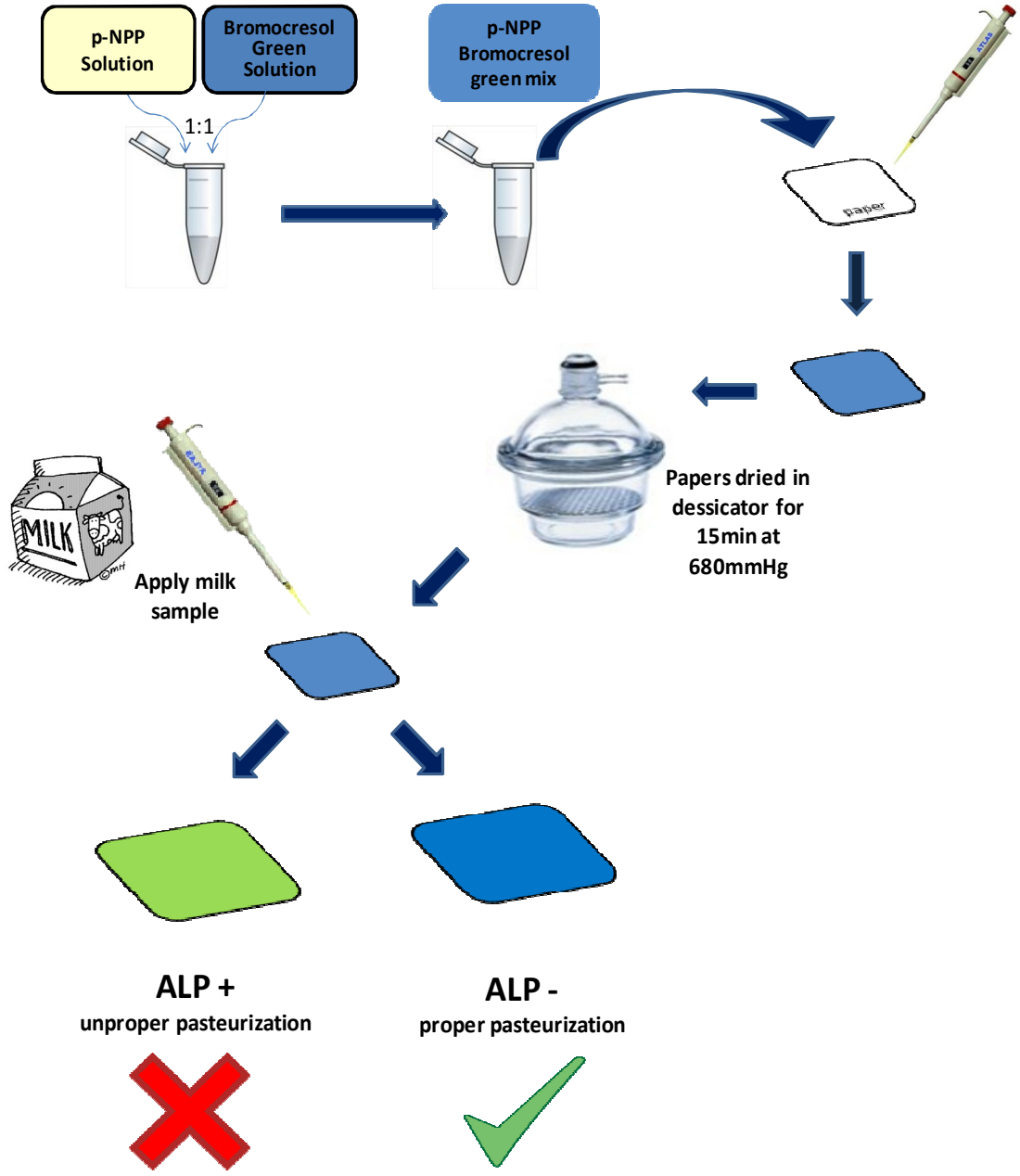

Figure 2.1. The representative figure of the construction of paper bioreporter

2.2.1.1 Preparation of Substrate Solution

For the construction of paper based bioreporter the optimization studies were performed by dissolving of p-NPP in three different buffers each of which were prepared at four different pH values. The 0.1 M glycine, 0.1 M sodium carbonate and 1.0 M diethanolamine buffers each of which was at pH 9.0, 9.5, 10.0 and 10.5 were used and the concentration of 2 mg/mL of p-NPP was selected as stock substrate concentration. The concentration of 2 mg/mL of p-NPP was prepared by dissolving 2.9 mg of p-NPP sodium salt in 1 mL of corresponding buffer. Substrate solutions were prepared freshly before each set of experiment and protected from direct light by covering containers with aluminum foil.

2.2.1.2 Preparation of Chromogen Solution

Bromocresol green selected as chromogen was dissolved in two different buffers, 1 M Tris-HCl and 0.1 M Glycine buffers, at pH 9.0, 9.5, 10.0 and 10.5. As a stock solution 2 mg/mL of bromocresol green was used for optimization studies. Chromogen solutions were prepared freshly before each experiment.

2.2.1.3 Preparation of Enzyme Solutions

The stock alkaline phosphatase enzyme solution was prepared in UHT milk at concentration of 100 units per mL. The concentration range of 0.1-10 U/mL alkaline phosphatase milk samples were prepared by dilution of stock enzyme solution with UHT milk.

2.2.1.4 Absorption on Support Materials

Freshly prepared p-NPP and bromocresol green solutions were mixed as 1:1 ratio prior to absorption on Whatman filter paper pieces (1cmX1cm) and 15 µL of this

mixture was applied on each piece of papers. As a control papers absorbed with only bromocresol green solution were prepared. All absorbed papers were dried in dessicator under vacuum at 680 mmHg for 15 min.

2.2.1.5 Substrate Optimization Studies

To specify the optimum substrate concentration for bioreporter construction 0.1 M glycine buffer with 1.0 mM magnesium chloride and 1.0 mM zinc chloride, 1.0 M diethanolamine buffer and 0.1 M sodium carbonate buffer were prepared at pH 9, 9.5, 10 and 10.5. The substrate stock solutions were prepared by dissolving 2 mg/mL of p-NPP in these buffers and diluted into 1 mg/mL and 0.5 mg/mL with corresponding buffers. During substrate optimization studies the chromogen concentration was kept constant at 0.1 mg/mL of bromocresol green in 1 M Tris-HCl buffer (pH 8.0). p-NPP solutions prepared in different buffers at selected pH values were mixed with bromocresol green solution and then absorbed to papers. After drying under vacuum color formation on papers following milk treatment with varying alkaline phosphatase concentrations were observed.

2.2.1.6 Chromogen Optimization Studies

For the determination of optimum chromogen concentration 2mg/mL bromocresol green was dissolved in 1.0 M Tris-HCl buffer and 0.1 M Glycine buffer at pH 8, 8.5, 9 and 9.5. The stock solutions of bromocresol green were diluted with these buffers to final concentrations of 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL. During the optimization studies of bromocresol green, p-NPP concentration was kept constant at 1 mg/mL in 0.1 M glycine buffer with 1.0 mM magnesium chloride and 1.0 mM zinc chloride. Papers were prepared by mixing substrate and chromogen solutions (1:1 ratio) and transferred to papers. Milk samples with different alkaline phosphatase concentration were applied on dried papers.

2.2.2 The Effect of Sample Temperature on Bioreporter Response

In order to determine the response of bioreporter to milk samples at different temperatures Whatman papers were prepared with 1 mg/mL p-NPP in 0.1 M glycine buffer at pH 9.5 and 0.5 mg/mL bromocresol green in 1.0 M Tris-HCl buffer at pH 9.5. Milk samples containing 0.1, 0.5, 1.0 and 5.0 unit per mL of alkaline phosphatase were stored at room temperature, 4°C and 37°C for 1 hour and then were tested against changes in response of papers. As control, UHT milk and glycine buffer were also applied to papers.

2.2.3 The Effect of Sample pH on Bioreporter Response

The pH of milk samples containing 0.1, 0.5 and 1.0 units per mL of alkaline phosphatase were adjusted to values between 5 and 6.5. These samples were then applied to papers prepared with optimized substrate and chromogen solutions and the results were analyzed. The UHT milk and glycine buffer were used as control.

2.2.4. The Response of Bioreporter to Different Milk Samples

2.2.4.1 Milk Samples from Different Animal Sources

In order to control the effectiveness of bioreporter against different sources of milk papers were prepared according to the result of optimization studies and responses of papers were tested with milk samples from sheep, goat and cattle.

2.2.4.2 Lipid Free Milk Samples

Milk samples from cattle, goat and sheep were centrifuged at 5000 rpm for 5 min at 25°C and the top lipid layers were removed. The result of lipid free milk samples were tested in order to observe the response of bioreporter. For the control of

negative responses, the lipid of UHT milk was removed and applied onto bioreporter.

2.2.4.3 Milk Samples from Different Location in Turkey

The constructed papers were tested against to milk samples from Bozova, Konya, Burdur, Kırıkkale, Varollar, Çamlık, Kutludüğün and Yahyalı. The control of this experiment was performed with commercial UHT milk.

2.2.5 Heat Treatment of Milk Samples

The heat treatment was applied to the standard and raw milk samples at 50°C and 60°C for 30 min in water bath. At 10 min interval samples were taken and tested for bioreporter response.

2.2.6 Addition of Raw Milk to Pasteurized Milk

To determine the contamination of pasteurized end product with raw milk, certain amount of raw milk sample from Kırıkkale were added to pasteurized milk. The detection of raw milk added to pasteurized milk at range of 0.1 % to 90 % was tested with prepared papers.

2.2.7 Different Support Materials

To find the most applicable support material for the construction of alkaline phosphatase bioreporter different materials were used in addition to Whatman filter paper. The cotton of ear cleaning rod and bandage were evaluated according to their response of UHT milk samples containing 0.5 U/mL of alkaline phosphatase. As a control UHT milk was also tested.

2.2.8 Shelf Life Study of Bioreporter

According to the optimization studies papers were prepared with 1 mg/mL p-NPP in 0.1 M glycine buffer at pH 9.5 and 0.5 mg/mL bromocresol green in 1.0 M Tris-HCl buffer at pH 9.5. They were stored in boxes covered with aluminum foil at 4°C and room temperature for 70 days. At 10 days interval the response of bioreporter were tested with milk sample of 0.5 units per mL alkaline phosphatase activity.

2.2.9 The Enzymatic Assay of Alkaline phosphatase in Milk

For plotting the standart alkaline phosphatase curve, p-NPP solution was prepared at a final concentration of 1 mg/mL in 0.1 M glycine buffer with 1.0 mM magnesium chloride and 1.0 mM zinc chloride at pH 9.5. As a blank solution, 2.9 mL of p-NPP solution was mixed with 0.1 mL of UHT milk and stored at room temperature for 10 min. Test samples were also prepared by mixing 2.9 mL of p-NPP solution with 0.1 mL of milk samples with alkaline phosphatase concentration ranging from 0.1 to 10 U/mL. Test samples were stored at room temperature for 10 min and after storage the absorbance values of each sample at 405 nm were monitored against blank solution for 5 min. The units of enzyme solutions were determined with the following equation (Dilution factor; df, Millimolar extinction coefficient of p-nitrophenol at 405 nm; 18.5, Volume of enzyme used; 0.1mL and volume of assay; 3 mL).

$$\text{Units/mL enzyme} = \frac{(\Delta A_{405nm}/\text{min Test} - \Delta A_{405nm}/\text{min Blank})(3)(df)}{(18.5)\,(0.1)}$$

One unit will hydrolyze 1.0 µmole of p-nitrophenyl phosphate per minute at pH 9.5 at room temperature.

2.2.10 Summary of The Study

Table 2.1 The parameters tested in the construction studies of alkaline phosphatase bioreporter

Parameters in Construction Studies			
	Buffers	pH	Concentrations
Substrate (p-NPP)	Sodium carbonate 0.1M (pKa : 10.33) Diethanolamine Buffer 1.0 M (pKa : 9.50) Glycine Buffer 0.1 M (pKa : 9.78)	9.0 9.5 10.0 10.5	2 mg/mL 1 mg/mL 0.5 mg/mL
Chromogen (Bromocresol green)	Glycine Buffer 0.1 M (pKa : 9.78) Tris-HCl Buffer 1.0 M (pKa : 8.06)	9.0 9.5 10.0 10.5	2 mg/mL 1 mg/mL 0.5 mg/mL 0.1 mg/mL

Table 2.2 The parameters in milk samples tested for the response of bioreporter

	Sample pH Effect	Sample Temperature Effect	Animal Source	Location in Turkey	Application of Heat	Addition of Raw Milk to UHT (%)
Milk Sample	5.0 5.5 6.0 6.5	4˚C RT 37˚C	Cattle Sheep Goat	Bozova Konya Burdur Kırıkkale Varollar Çamlık Kutludüğün Yahyalı	50˚C 60˚C	1 5 10 20 30 40 50 60 70 80 90

Table 2.3 The Support Materials and Stability Conditions of Bioreporter

	Support Material	Condition for Stability Test	
Bioreporter	Whatman Filter Paper Cotton Bandage	4˚C	RT

2.3 Green Color Intensity Ratio Analysis

The quantitative analysis of color observed on papers after the application of different milk samples was performed by OBİTEK Obicolor Master Software. The papers were constructed according to the results of optimization studies of substrate and chromogen. The papers with optimized substrate and chromogen concentrations were dried in vacuum desicator at 680 mmHg for 15 min. The milk samples were applied to papers and after drying for 5 min the papers were scanned by Hewlett-Packard PSC 1400 with parameters of 24 bits. The images of papers were acquired with a flatbed scanner at 8 bit dynamic range (256 levels). The formation of green color on papers was analyzed with OBİTEK ObiColor Master Software (Figure 2.2).

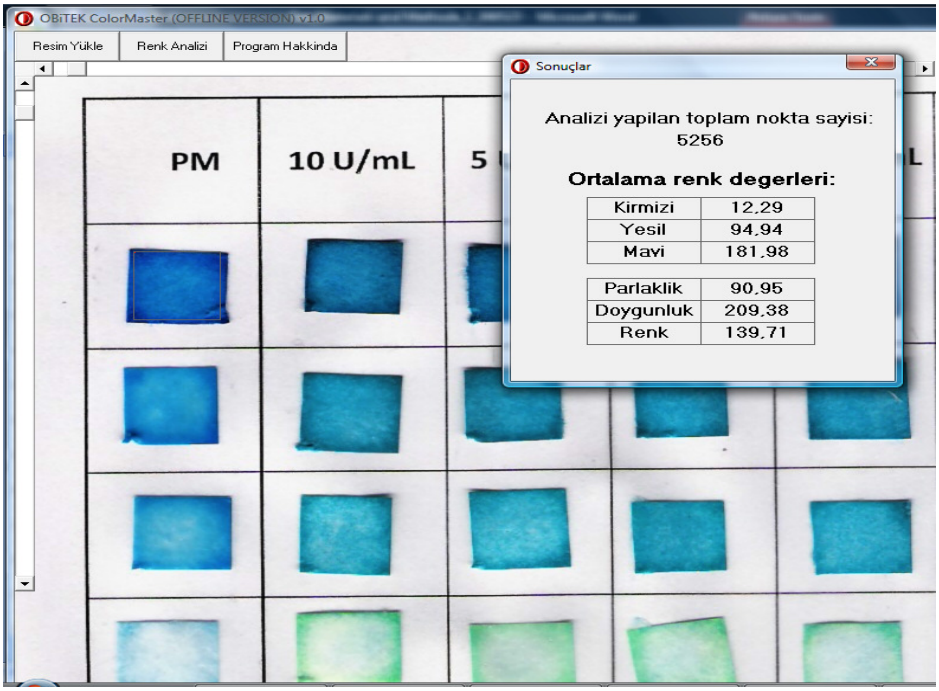

Figure 2.2.Color intensity analysis performed by OBİTEK ObiColor Master Software.

OBİTEK ObiColor Master Software is used for the measurement of RGB (red, blue and green) color intensity. It measures the color intensity between the value of 0 and 255 corresponding to pure black and pure white respectively. In our study, the green color formed on papers was measured by dividing the green color intensity value with the total color intensity value (red, blue and green) represented in the following equation. All the experiments were performed in triple sets.

Green Color Intensity Ratio: $\frac{I\ green}{(I\ red + I\ green + I\ blue)}$

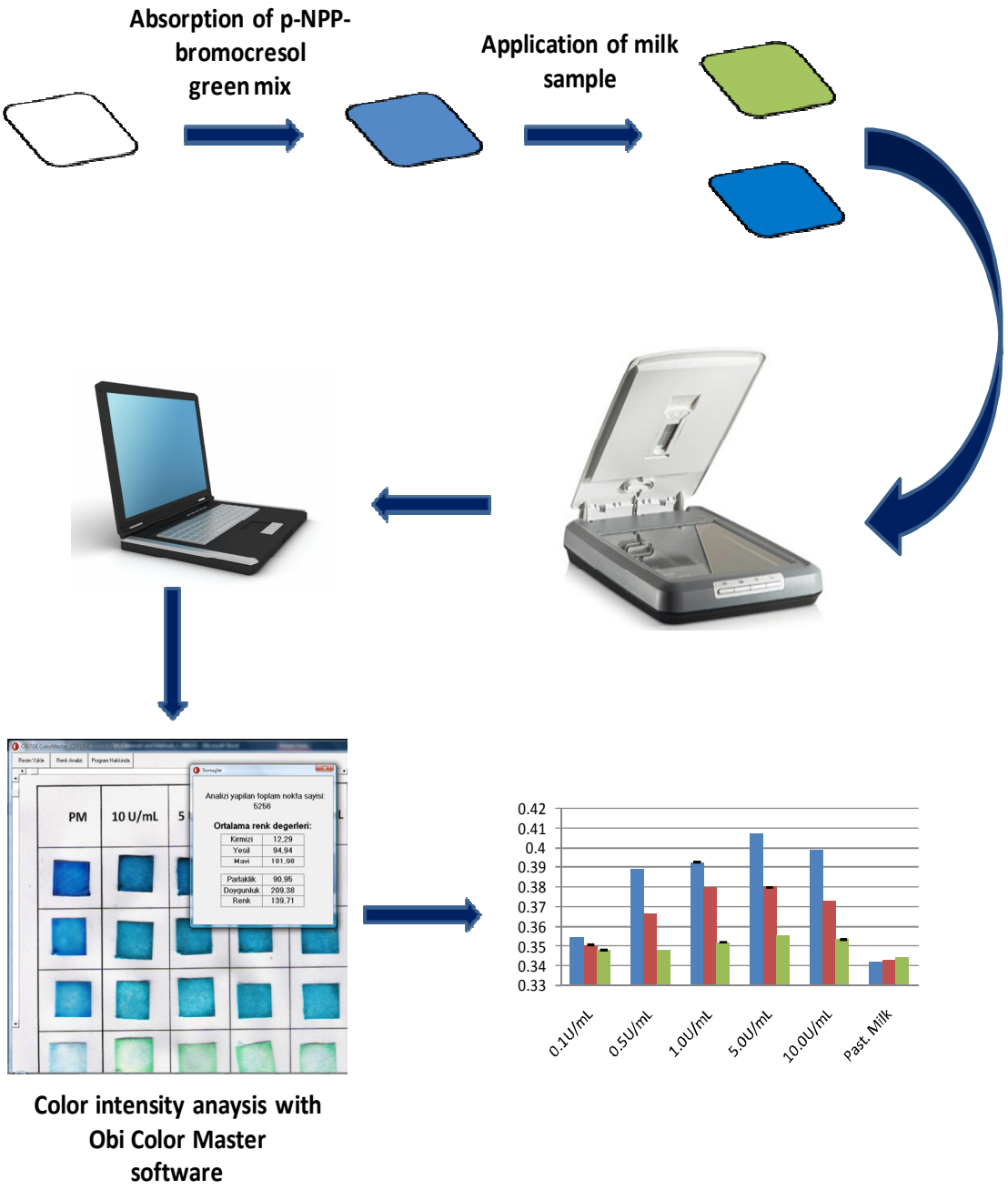

Figure 2.3 The representative figure of steps for green color intensity analysis

2.4 Statistical Analysis

For the statistical analysis of the color intensity the Minitab 15.0 software package was used. The mean values and standard error of means (SEM) of replicates were calculated and the variance in mean values of different treatments was evaluated in one way analysis of variance (ANOVA) at 95 % confidence interval. All of the mean and standard error of mean values are tabulated at tables in Appendix B.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization Studies

The construction of alkaline phosphatase bioreporter for validation of pasteurization process began with a series of optimization studies. In order to determine the optimum substrate and chromogen concentration different buffers at different pH values were tested according to their buffering range. p-nitrophenlyphosphate and bromocresol green were used as the substrate and chromogen of alkaline phosphatase bioreporter respectively. The response of bioreporter to milk samples containing alkaline phosphatase within a concentration range of 0.1-10 U/mL was observed as a color change. In the optimization studies, alkaline phosphatase solutions were prepared in UHT milk since buffers could not mimic the milk completely because of its complexity (Kelly & Fox, 2006). Therefore milk samples containing known amount of alkaline phosphatase were used to observe the response of bioreporter. Based on visual inspection and green color intensity ratio analysis the optimum concentrations of p-NPP and bromocresol green were determined for further studies.

3.1.1 Optimization Studies of Substrate

In the optimization studies of p-NPP sodium carbonate , diethanol amine and glycine buffers were used in order to determine the optimum p-NPP

concentration immobilized to papers. These buffers were prepared at pH values of 9.0, 9.5, 10.0 and 10.5 within their buffering ranges. The stock solution of p-NPP was prepared as 2 mg/mL in these buffers at each pH value. 1 mg/mL and 0.5 mg/mL p-NPP solutions were prepared by diluting the stock solutions in corresponding buffers. Throughout the optimization studies of substrate, chromogen concentration was kept constant. Bromocresol green was used as 0.1 mg/mL in 1 M Tris-HCl buffer at pH 8.0. Each p-NPP solution was mixed with bromocresol green solution at 1:1 ratio and these mixtures were absorbed to papers. Milk samples containing different amount of alkaline phosphatase were applied to papers to observe color changes.

3.1.1.1 p-NPP in Sodium Carbonate Buffer

In the study stock solutions of 2 mg/mL of p-NPP were prepared in 0.1 M sodium carbonate buffer at pH 9.0, 9.5, 10.0, and 10.5. The other concentrations of substrate solution (1 mg/mL and 0.5 mg/mL) were prepared at each pH value. The effect of substrate concentration in sodium carbonate buffer at each pH value was observed visually. Figure 3.1 represents the results of sodium carbonate buffer at pH 9.0.

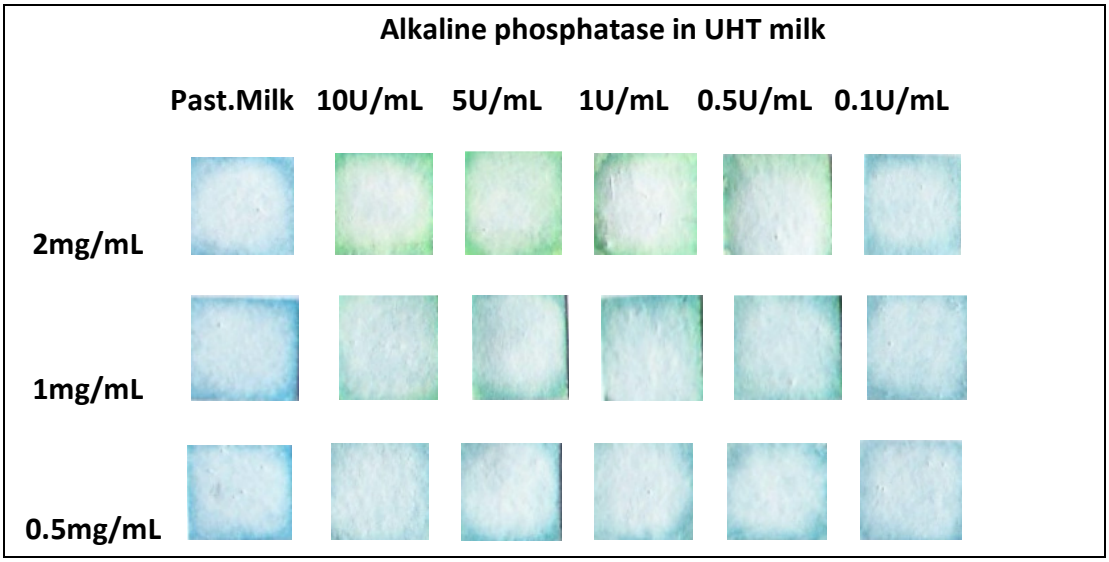

Figure 3.1. The effect of p-NPP concentration dissolved in 0.1 M sodium carbonate buffer at pH 9.0 to the response of bioreporters

As shown in Figure 3.1 the green color intensity increases with increase in the concentration of alkaline phosphatase in milk and this increase is obvious in papers prepared with 2 mg/mL p-NPP concentration.

In addition, the green color intensity ratio of these bioreporters were analysed (Figure 3.2). The papers prepared with 0.5 mg/mL of p-NPP have not shown any increase in the green color against increasing amount of alkaline phosphatase in milk while papers absorbed with 1 mg/mL and 2 mg/mL of p-NPP have shown. However this increase in green color intensity ratio at these papers has not changed consistently.

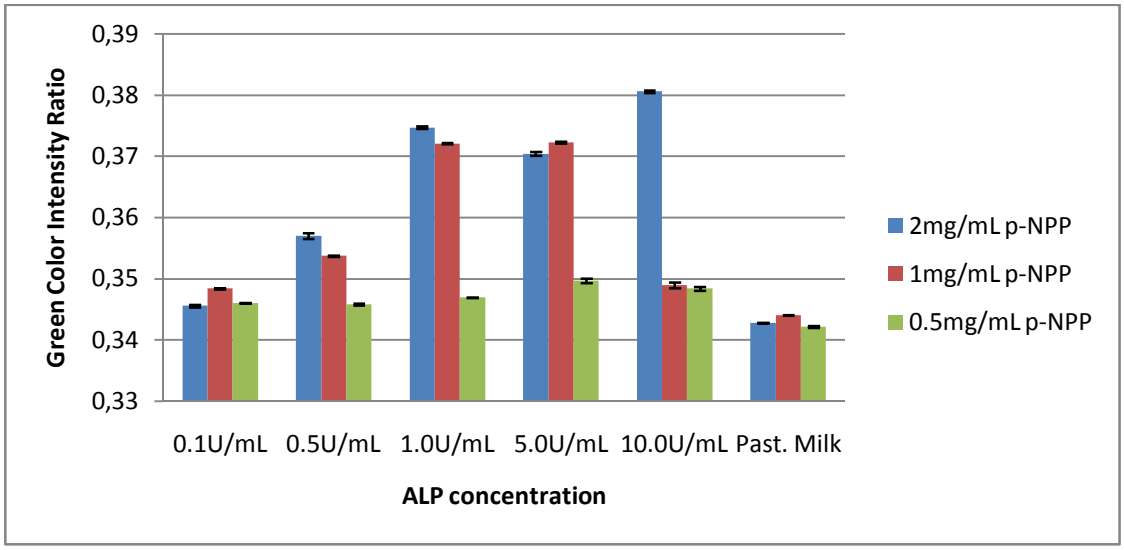

Figure 3.2. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M sodium carbonate buffer at pH 9.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.1 in Appendix B.

According to the results shown in Figure 3.1 and 3.2, sodium carbonate buffer at pH 9.5 was tried to prepare substrate solutions at three different concentrations. The response of the bioreporters to milk samples which is the formation of green color upon the application of milk sample containing alkaline phosphatase were analysed visiually and the color intensity values were obtained. The color change in the papers at pH 9.5 showed similar results with papers at pH 9.0.

As shown in Figure 3.3, the increase in green color intensity has been seen obviously in papers absorbed with 2 mg/mL of p-NPP while the enzyme concentration increases in milk.

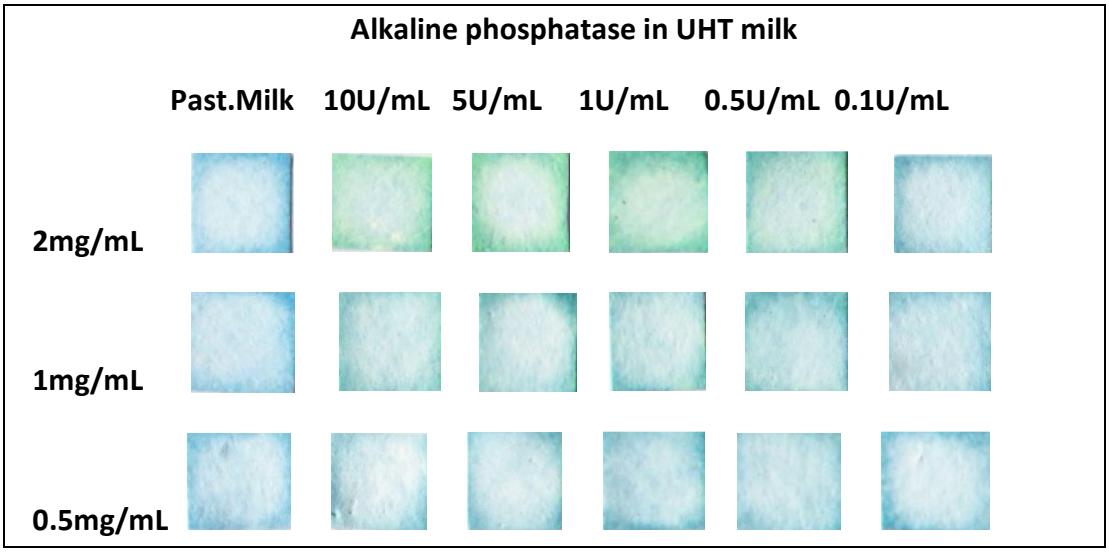

Figure 3.3. The effect of p-NPP concentration dissolved in 0.1 M sodium carbonate buffer at pH 9.5 to the response of bioreporters.

This result has been also supported with color intensity analysis. (Figure 3.4). The papers prepared with 2 mg/mL of p-NPP in sodium carbonate buffer at pH 9.5 have shown significant increase in green color with increasing enzyme concentration in milk.

However, alkaline phosphatase concentration of 10.0 U/mL has brought about a decrease in color intensity value at this concentration of substrate. The papers prepared with other concentration of p-NPP has not responsed appropiately. As a results, other pH values of sodium carbonate buffer was studied in the next experiments.

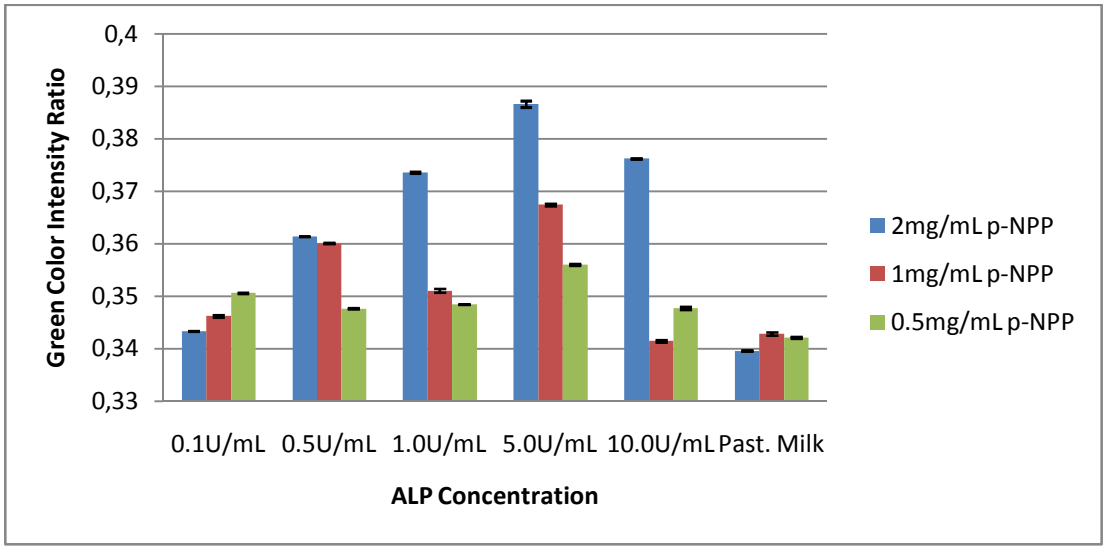

Figure 3.4. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M sodium carbonate buffer at pH 9.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.2 in Appendix B.

The papers prepared with p-NPP solution in sodium carbonate buffer at pH 10.0 have given the the responses shown in Figure 3.5 to milk samples containing different units of alkaline phosphatase.

As the visual and color intensity analysis of papers prepared in substrate solution at pH 9.0 has been compared with papers at pH 10.0 , same results have obtained. The p-NPP solution at 2 mg/mL and 1 mg/mL concentration have represented significant responses although these responses are not consistent (Figure 3.6).

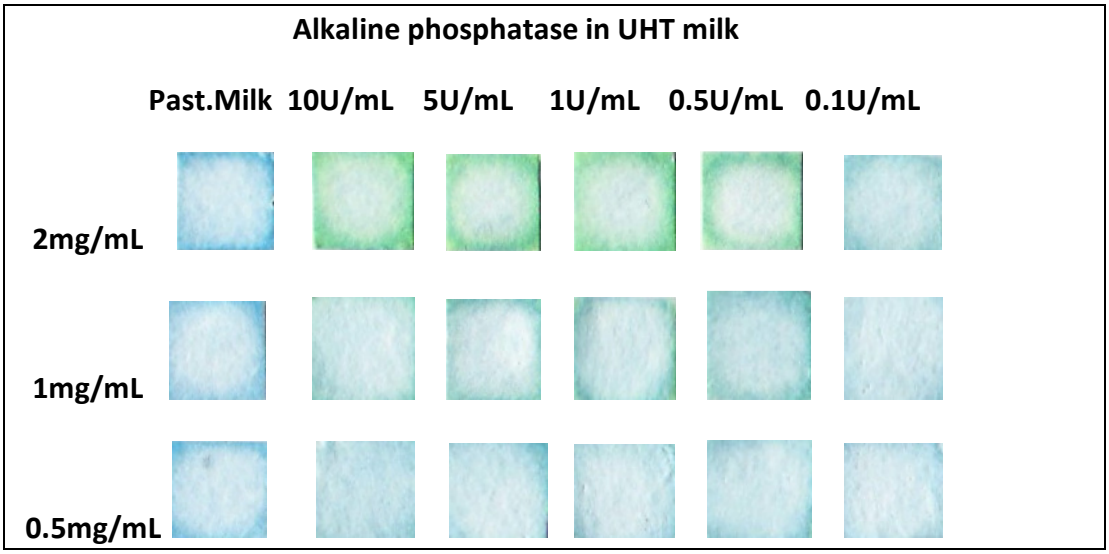

Figure 3.5. The effect of p-NPP concentration dissolved in 0.1 M sodium carbonate buffer at pH 10.0 to the response of bioreporters

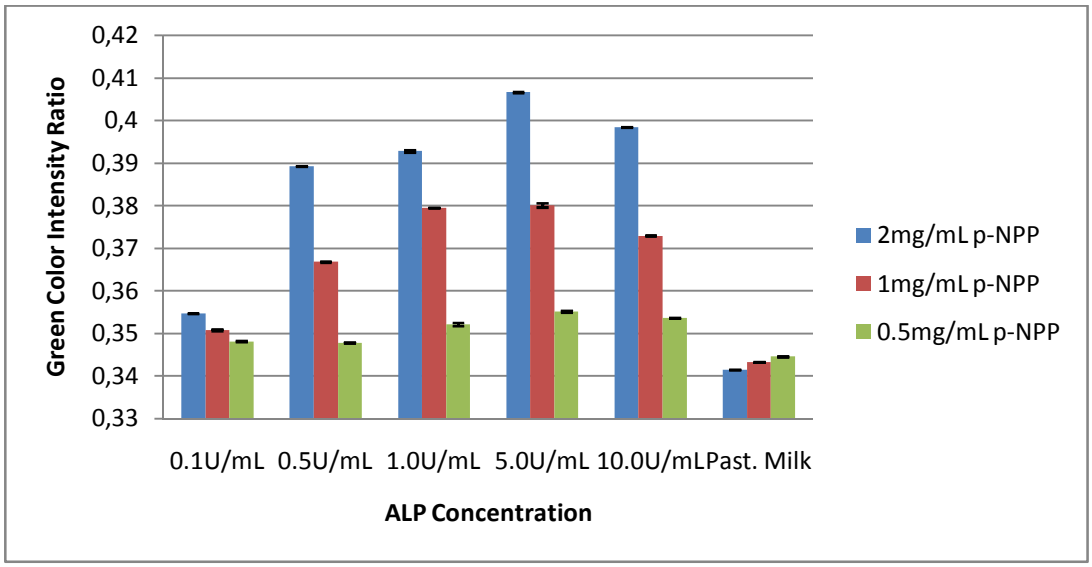

Figure3.6. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M sodium carbonate buffer at pH 10.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.3 in Appendix B.

The sodium carbonate buffer at pH 10.5 was tested finally for the preparation of substrate solution since proper results were not taken from previous studies performed at pH 9.0, 9.5 and 10.0. However, as shown in the Figure 3.7 and 3.8 representing the responses of papers to milk samples and the color intensity analysis respectively, sodium carbonate buffer at each pH value has given similar results which are not applicable for the construction of alkaline phosphatase bioreporter. Therefore, sodium carbonate buffer was not selected for the preparation of substrate solution.

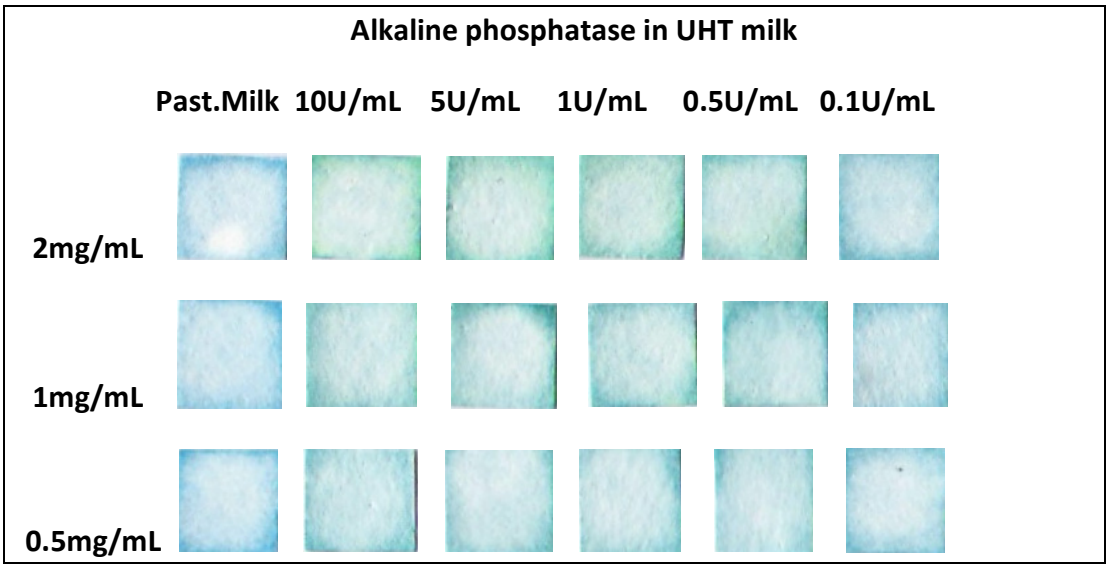

Figure 3.7. The effect of p-NPP concentration dissolved in 0.1 M sodium carbonate buffer at pH 10.5 to the response of bioreporters

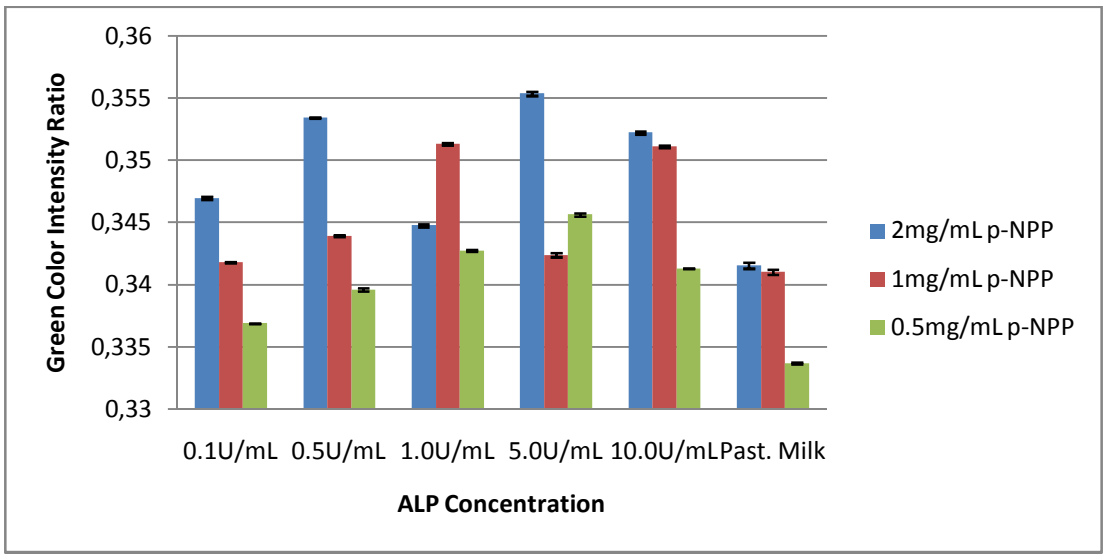

Figure 3.8. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M sodium carbonate buffer at pH 10.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.4 in Appendix B.

3.1.1.2 p-NPP in Diethanolamine Buffer

The next set of experiment was preformed in 1 M diethanolamine buffer at four different pH values. The responses of papers to milk samples with different concentration of alkaline phosphatase were analysed by visually and numerical values of the color changes were used to support the visual analysis. The following figures represent the color changes of papers prepared with different p-NPP concentration at pH 9.0 of diethanolamine buffer after they were tested with milk samples.

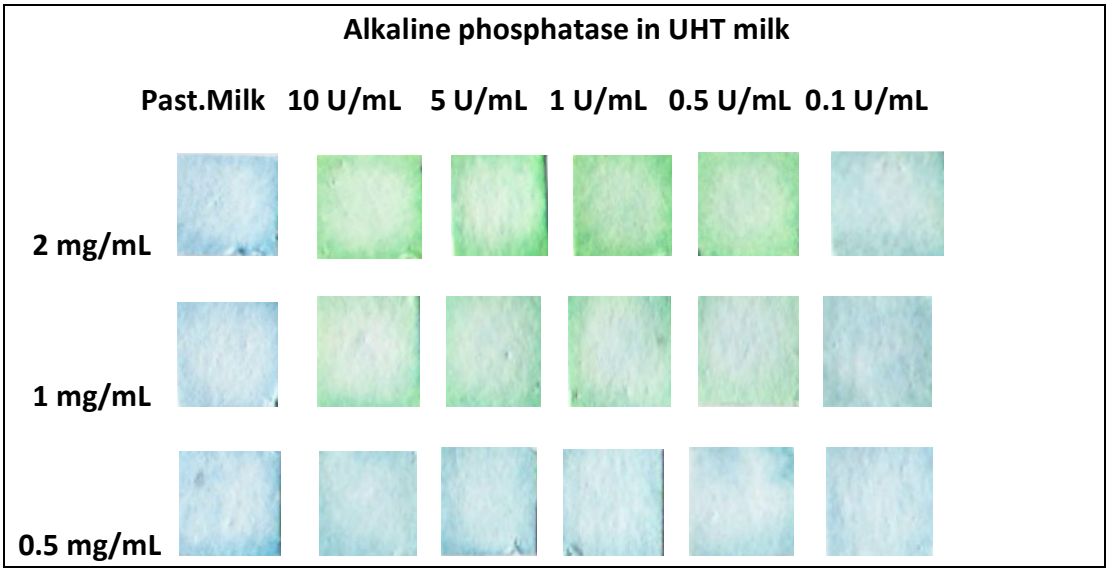

Figure 3.9. The effect of p-NPP concentration dissolved in 1.0 M diethanolamine buffer at pH 9.0 to the response of bioreporters

According to the visual analysis (Figure 3.9) the changes in the color after the application of milk samples containing a range of alkaline phosphatase concentration have been similar in sodium carbonate and diethanolamine buffer. However, the color intensity analysis has represented different result. As shown in the figure below (Figure 3.10) the response of papers at 1 mg/mL of p-NPP concentration has been evaluated as appropriate when compared to the other concentration of substrate. The consistant increase in green color intensity has been observed to the alkaline phosphatase concentration of 5.0 U/mL . However, at the concentration of 10.0 U/mL of alkaline phosphatase the numerical value of green color has been decreasing which is not an expected result. It should represent at least a value approximately same with value of 5.0 U/mL of enzyme concentration. This result can be explained by the failure in scanning process of scanner or in the measurement of green color value.

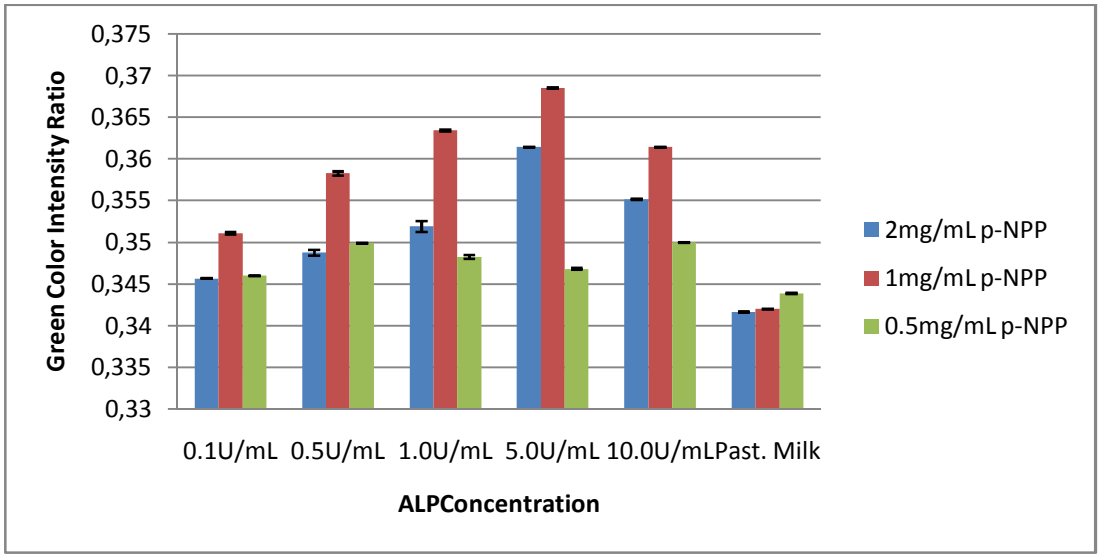

Figure 3.10. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 1.0 M diethanolamine at pH 9.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.5 in Appendix B.

Since appropriate results could not be taken from the pH value of 9.0, the next experiment was performed with diethanol amine buffer at pH 9.5. Although the increase in green color intensity has been observed with increase in the enzyme concentration by visual analysis (Figure 3.11), these results are not supported quantitatively by color analysis (Figure 3.12). The scattered data represented in the graph has demostrated that the diethanolamine buffer at pH 9.5 is unpractical for the construction of bioreporter.

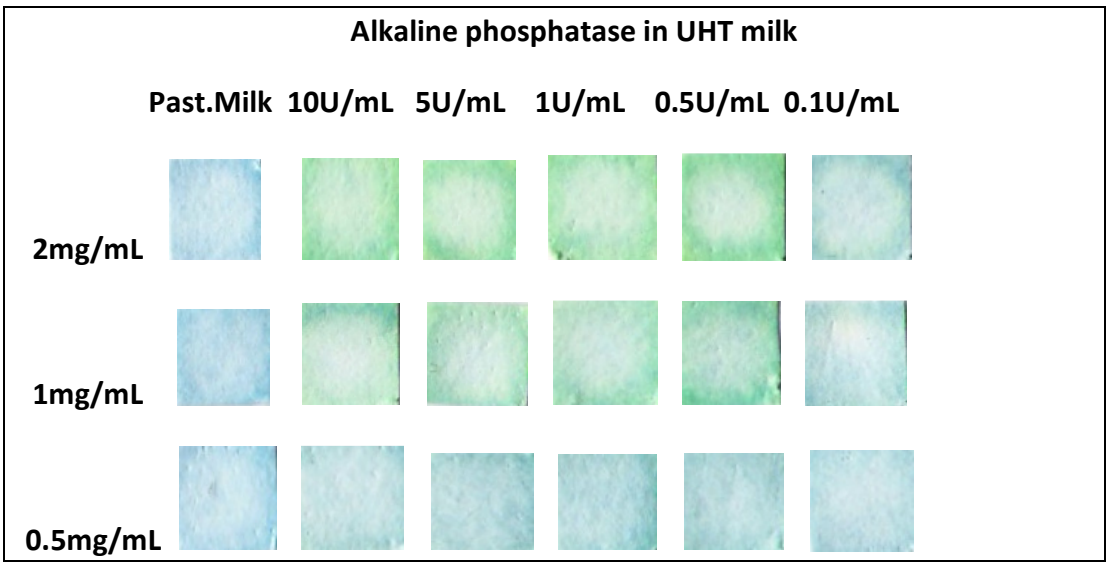

Figure 3.11. The effect of p-NPP concentration dissolved in 1.0 M diethanolamine buffer at pH 9.5 to the response of bioreporters

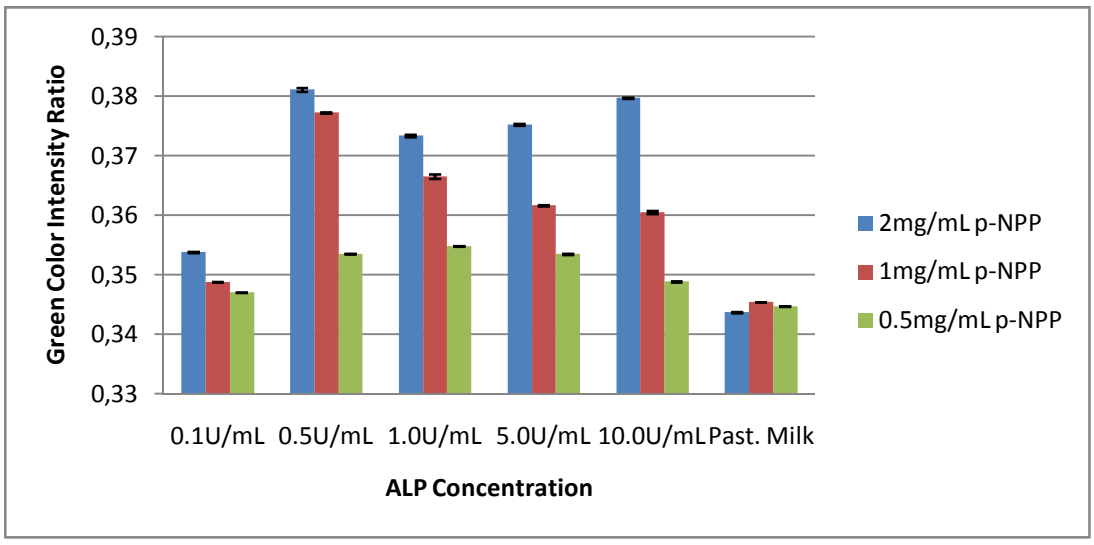

Figure 3.12. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 1.0 M diethanolamine buffer at pH 9.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.6 in Appendix B.

In consequence of impractical results, the pH value of substrate solution was increased to 10.0 and 10.5 to obtain a workable and expected result for the construction of alkaline phophatase bioreporter. The visual analysis were peformed by observing the formation of green color on papers after the application of milk samples. Figure 3.13 and Figure 3.15 represents the color changes on papers prepared with substrate solutions at pH 10.0 and 10.5, respectively.

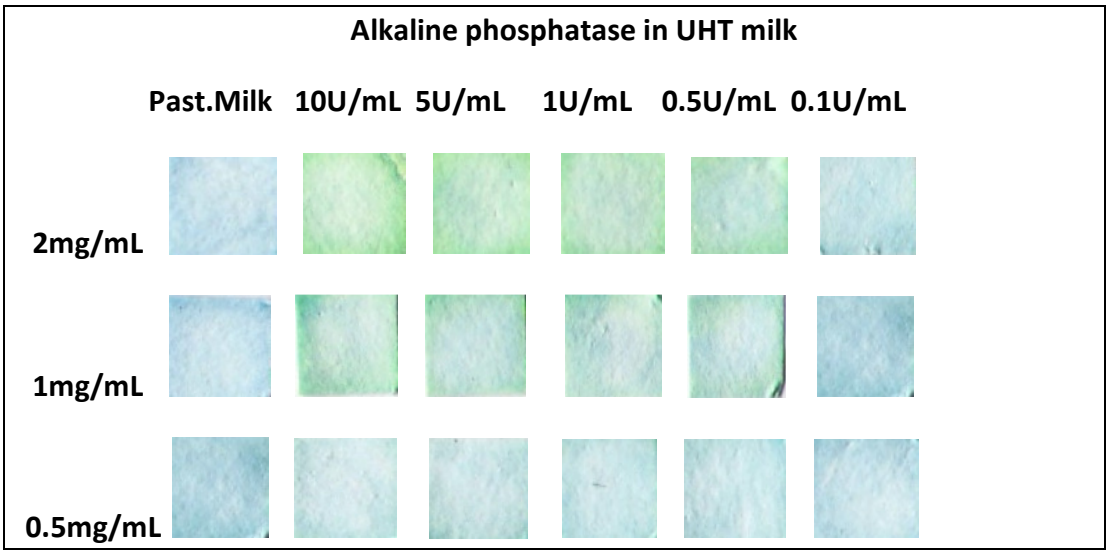

Figure 3.13. The effect of p-NPP concentration dissolved in 1.0 M diethanolamine buffer at pH 10.0 to the response of bioreporters.

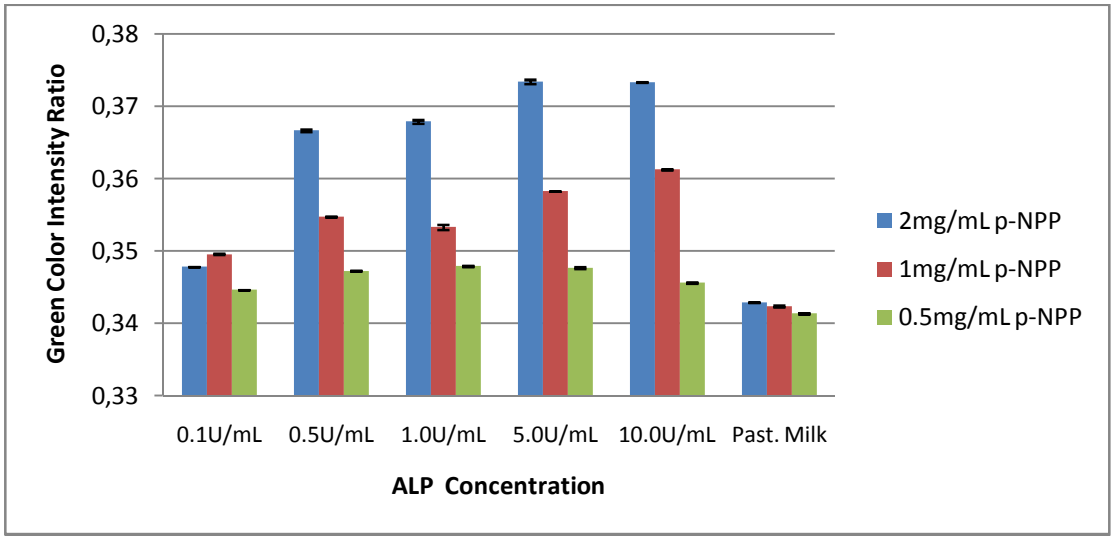

Figure 3.14. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 1.0 M diethanolamine buffer at pH 10.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.7 in Appendix B.

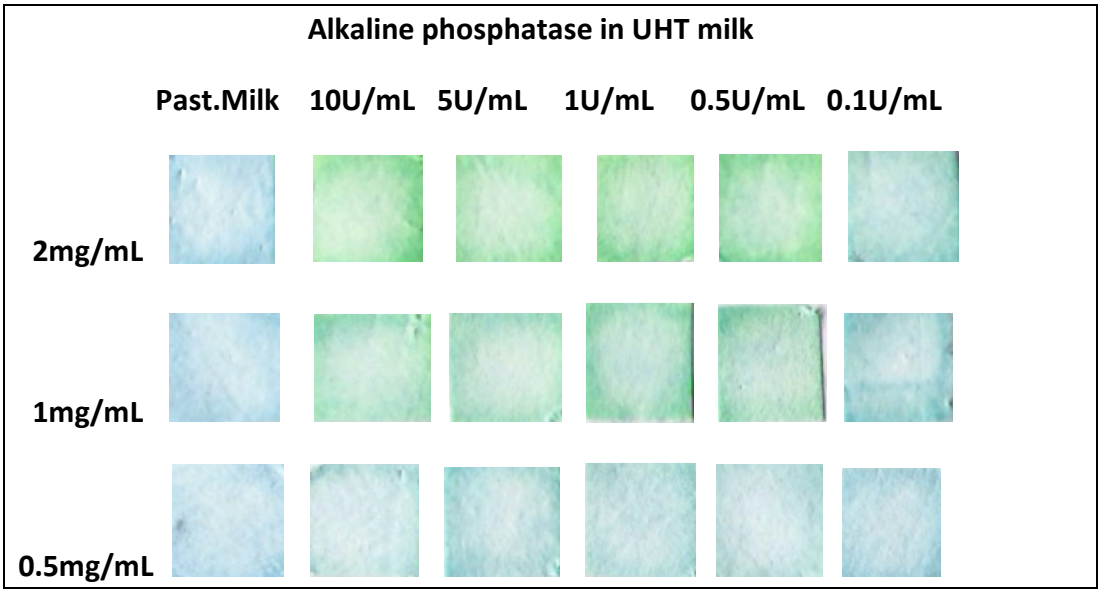

Figure 3.15. The effect of p-NPP concentration dissolved in 1.0 M diethanolamine buffer at pH 10.5 to the response of bioreporters.

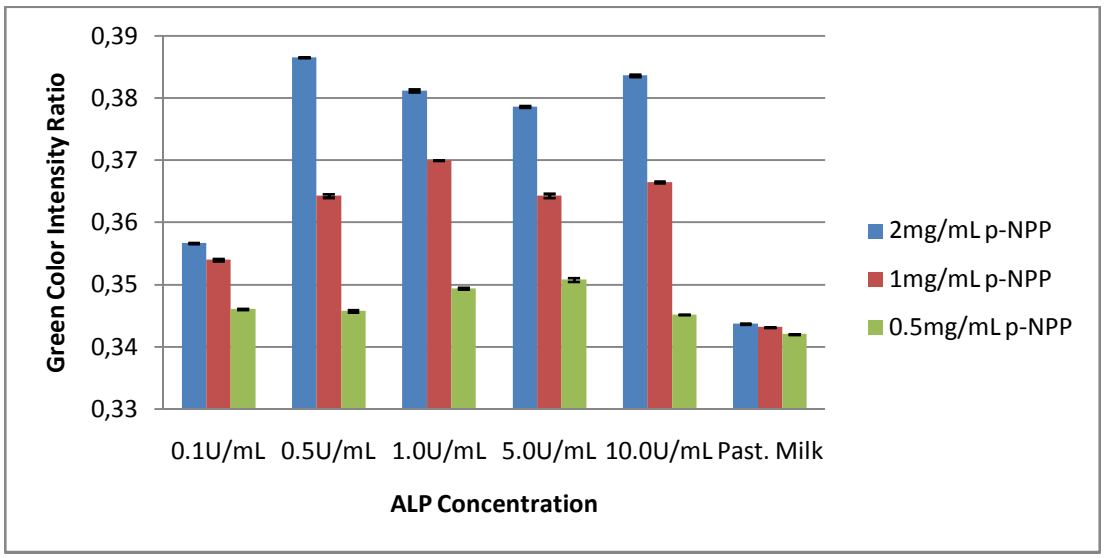

Figure 3.16. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 1.0 M diethanolamine at pH 10.5 Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.8 in Appendix B.

The quantitative data of papers prepared at pH 10.0 (Figure 3.14) and 10.5 (Figure 3.16) have shown approximately same results as compared with papers prepared at pH 9.5. The expected linear increase in green color intensity with increase in enzyme concentration were not achieved at both pH value.

According to the visual and color analysis results , the diethanol amine was not prefered for the buffer of the optimum substrate solution. In addtion to the impractical results the diethanol amine buffer was not selected for the substrate solution due to the irritant property of its content.

3.1.1.3 p-NPP in Glycine Buffer

Final optimization study of p-NPP was conducted with glycine buffer at four different pH values. In the course of the preparation of substrate solution 0.1 M

glycine buffer was supplemented with 1.0 mM magnesium chloride and 1.0 mM zinc chloride. According to studies the magnesium and zinc ions activates the alkaline phosphatase enzyme of *Bos taurus* (Strinson & Chan, 1987). Therefore, during the preparation of glycine buffer these ions were added in their salt forms. The stock solution of pNPP was prepared as 2 mg/mL at each pH value and solutions other concentration of substrate solutions were made from these stocks. Each p-NPP solution was mixed at 1:1 ratio with bromocresol green solution and absorbed to papers. Analysis was performed after the application of milk samples.

The response of papers prepared at each pH value of glycine buffer and color intensity analysis of the green color formation is shown in the following figures sequentially.

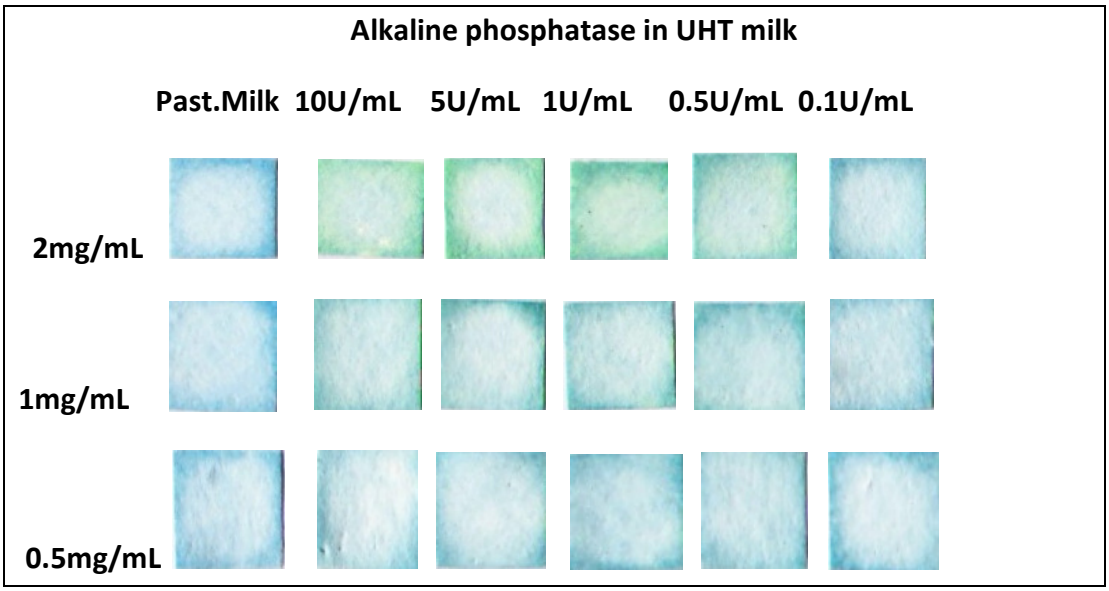

Figure 3.17. The effect of p-NPP concentration dissolved in 0.1 M glycine at pH 9.0 to the response of bioreporters.

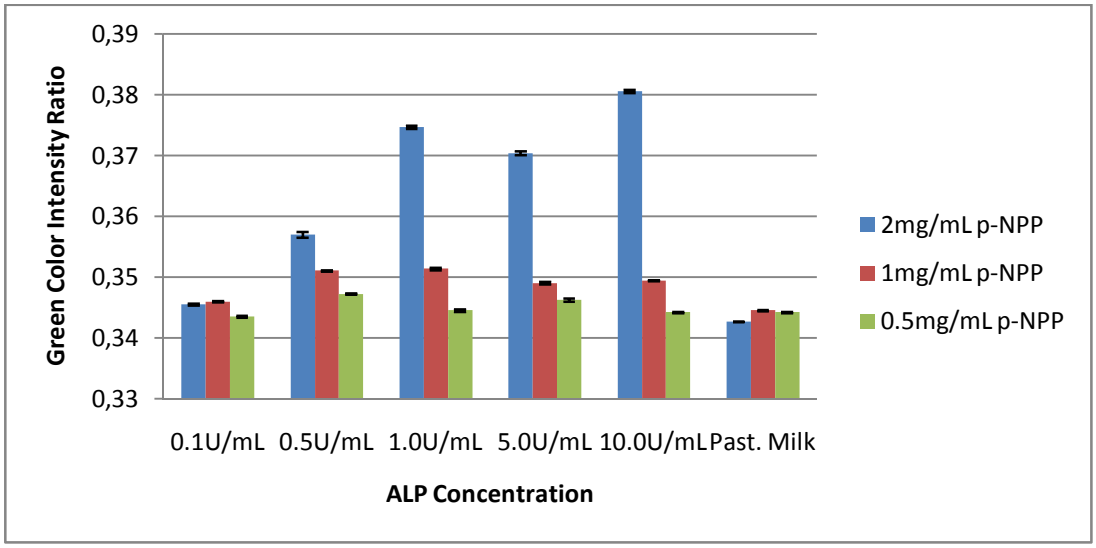

Figure 3.18. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M glycine buffer at pH 9.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.9 in Appendix B.

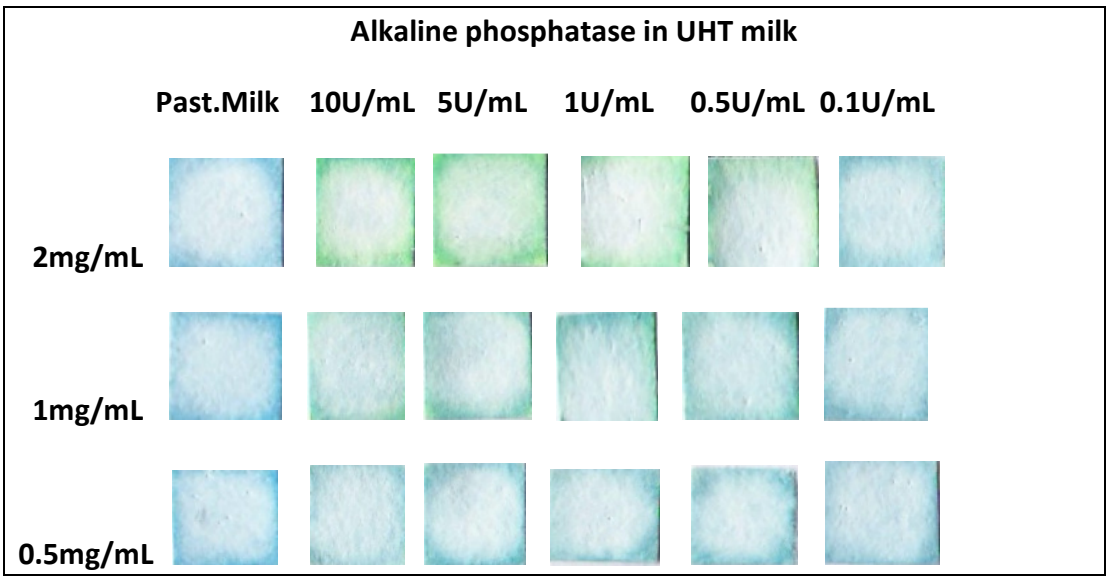

Figure 3.19. The effect of p-NPP concentration dissolved in 0.1 M glycine buffer at pH 9.5 to the response of bioreporters.

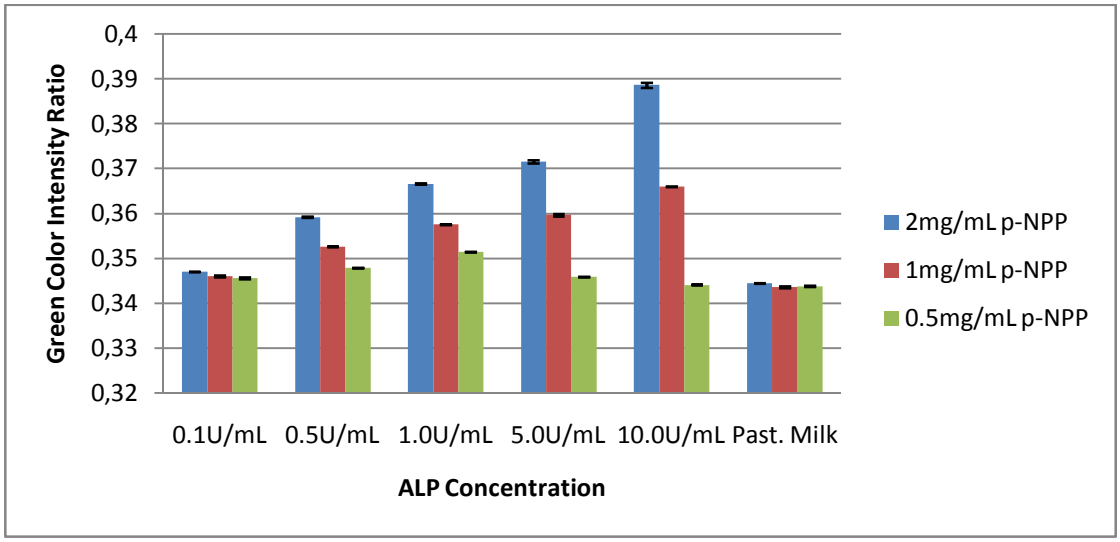

Figure 3.20. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M glycine buffer at pH 9.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.10 in Appendix B.

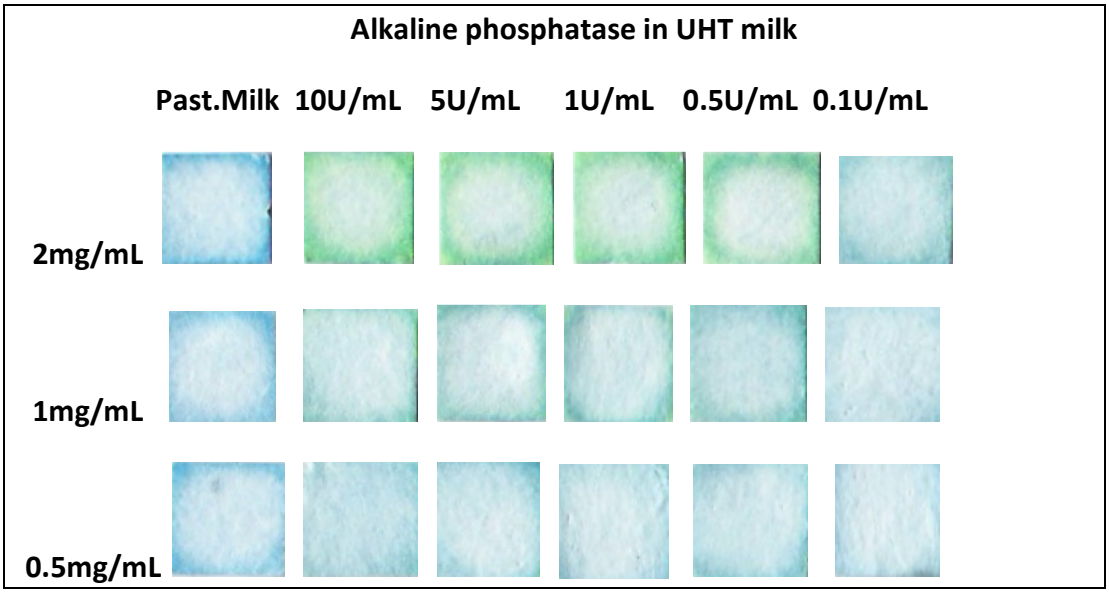

Figure 3.21. The effect of p-NPP concentration dissolved in 0.1 M glycine buffer at pH 10.0 to the response of bioreporters.

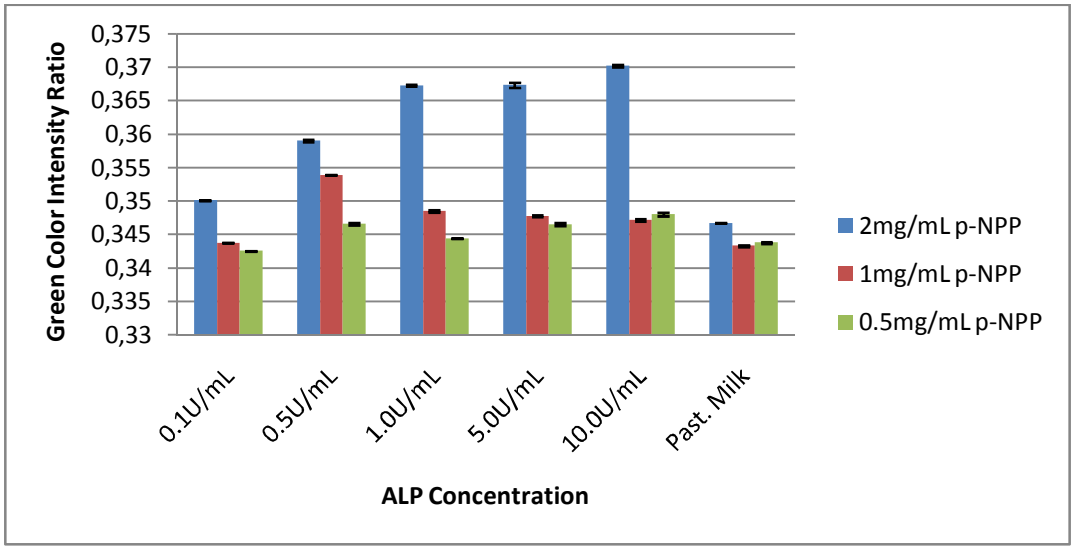

Figure 3.22. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M glycine buffer at pH 10.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.11 in Appendix B.

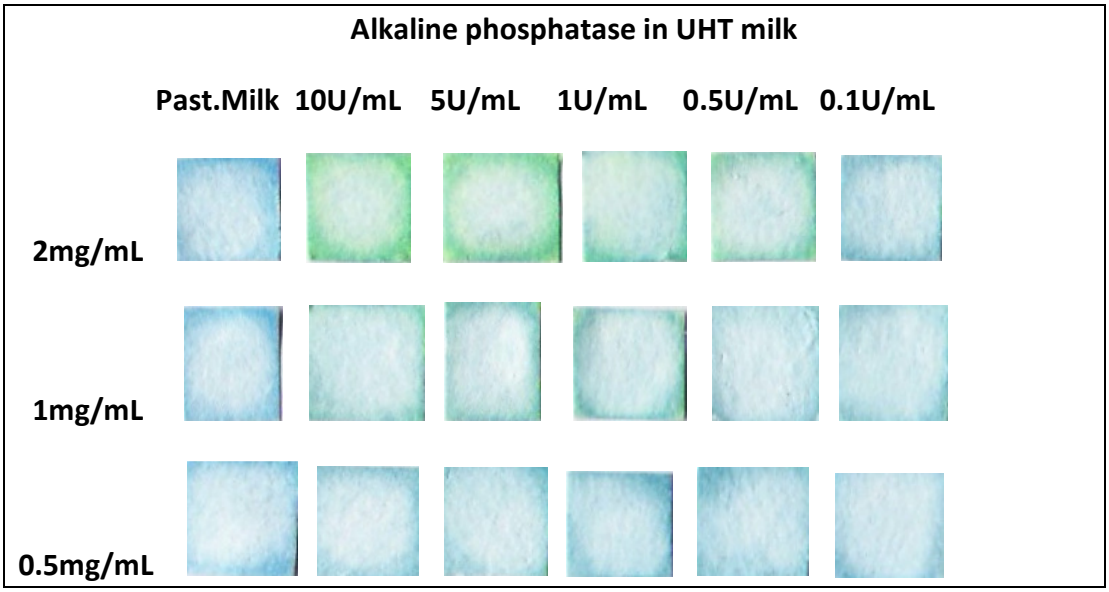

Figure 3.23. The effect of p-NPP concentration dissolved in 0.1 M glycine at pH 10.5 to the response of bioreporters.

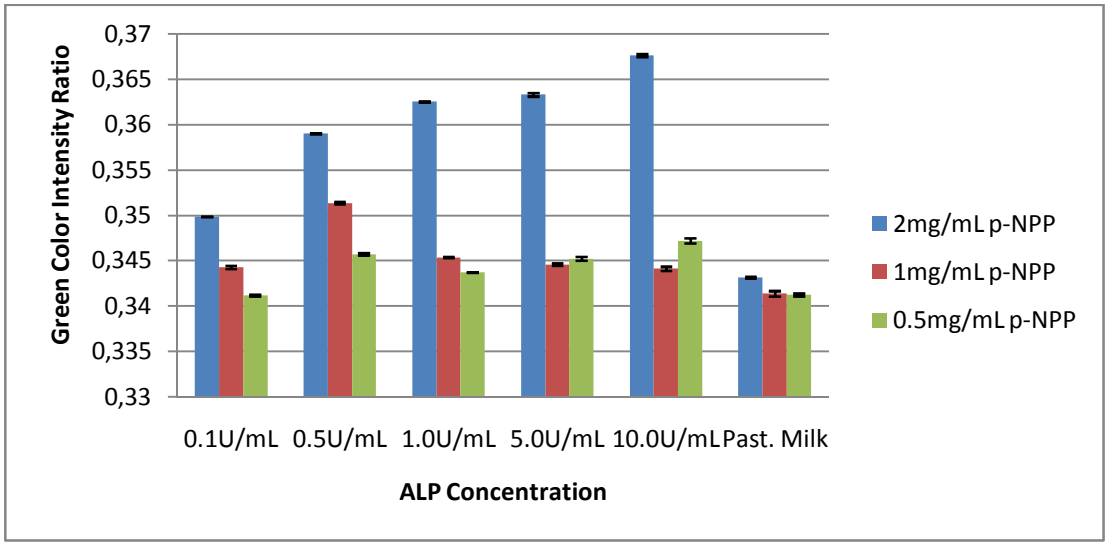

Figure 3.24. The analysis of green color intensity ratio of bioreporters prepared with different amounts of p-NPP in 0.1 M glycine buffer at pH 10.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.12 in Appendix B.

The papers prepared with glycine buffer at pH 9.0, 10.0 and 10.5 have been shown similar responses to milk samples containing a range of 0.1 U/mL to 10.0 U/mL of alkaline phosphatase with 0.5 mg/mL of p-NPP. Their numerical values have represented that only the substrate solution at 2 mg/mL of p-NPP show significant responses to increasing enzyme concentration. However, papers prepared with 2 mg/mL and 1 mg/mL of p-NPP at pH 9.5 have increasing green color value with increasing alkaline phosphatase concentration in milk. Both concentration of p-NPP in glycine buffer at pH 9.5 can be applicable as optimum substrate solution but in our study we selected 1 mg/mL of p-NPP solution in order to reduce the production cost of bioreporters. As a result, 1 mg/mL of p-NPP in glycine buffer at pH 9.5 was determined as an optimum solution of substrate for the construction of alkaline phosphatase bioreporter.

3.1.2 Optimization studies of chromogen

After the selection of substrate concentration , chromogen optimization studies were performed for the construction of alkaline phosphatase bioreporter. The chromogen selected for alkaline phosphatase, bromocresol green, was dissolved in 1.0 M Tris-HCl buffer and 0.1 M glycine buffer each of which were prepared at pH values of 8.0, 8.5, 9.0 and 9.5. The stock solutions of 2 mg/mL of bromocresol green were prepared at each pH values of these two buffers and they were diluted to 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL final concentration of bromocresol green solutions. During the studies for the optimization of chromogen the optimized p-NPP solution as 1 mg/mL in 0.1 M glycine buffer at pH 9.5 was used in chromogen-substrate mix solutions. The results were analysed based on the green color changes after the application of milk samples.

3.1.2.1 Bromocresol green in Glycine Buffer

As shown in the optimization studies of substrate, 1 mg/mL of p-NPP in 0.1 M glycine buffer at pH 9.5 was selected as the optimum substrate solution for the construction of alkaline phosphatase bioreporters. The optimization studies of chromogen, therefore, began with the use of 0.1 M glycine buffer at four different pH values for the preparation of bromocresol green solutions.

Figure 3.25 has shown the responses of papers absorbed with different concentration of bromocresol solutions at pH 8.0. The formation of green color due to the enzymatic activity between alkaline phosphatase and p-NPP has been observed clearly on papers prepared with 0.1 mg/mL of bromocresol green. At higher concentration of chromogen blue color of bromocresol green has prevented the appearance of green color.

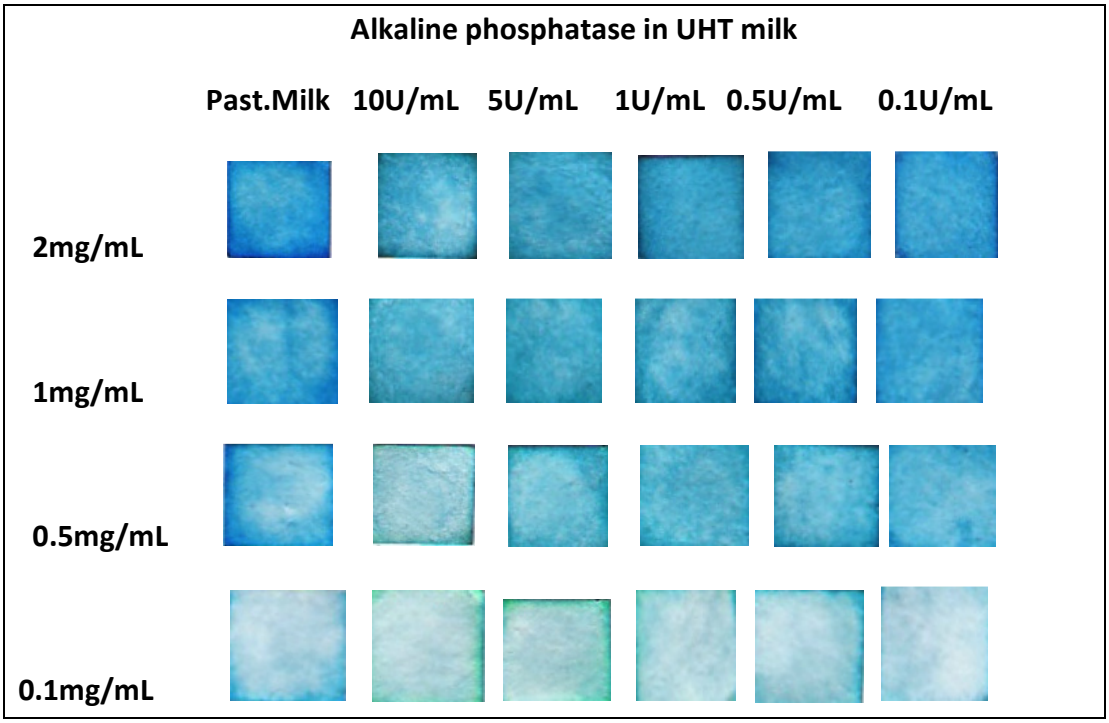

Figure 3.25. The effect of bromocresol green concentration dissolved in 0.1 M glycine buffer at pH 8.0 to the response of bioreporters

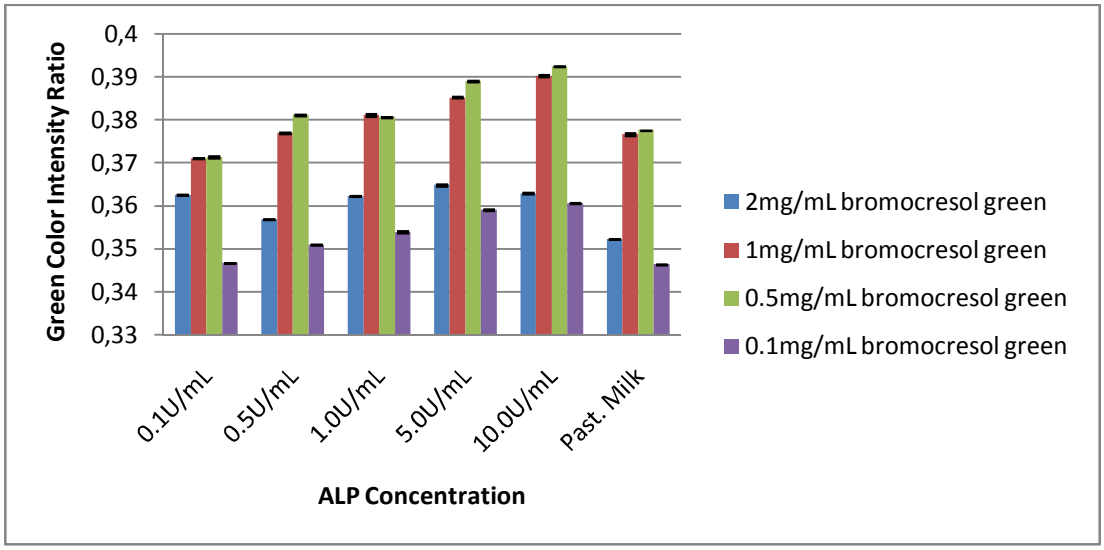

Figure3.26. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 0.1 M glycine buffer at pH 8.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.13 in Appendix B.

The numerical values of papers (Figure 3.26) have provided different results as compared with the visual analysis(Figure 3.25). The color intensity value of papers at 1 mg/mL and 0.5 mg/mL have shown significant differences from papers at other concentrations of bromocresol green. Our bioreporters are based on visual analysis and numerial values of color changes is not provided to the user of this bioreporter. The discrimination between the colors of papers after the application of milk samples is the aim of this bioreporter. The papers prepared with bromocresol green in glycine buffer at pH 8.0 has not shown a clear difference when increasing concentration of alkaline phosphatase in milk samples were applied to papers. Therefore , the pH of glycine buffer was increased to 8.5 for the next study and the results were analysed according to the aim of our bioreporter.

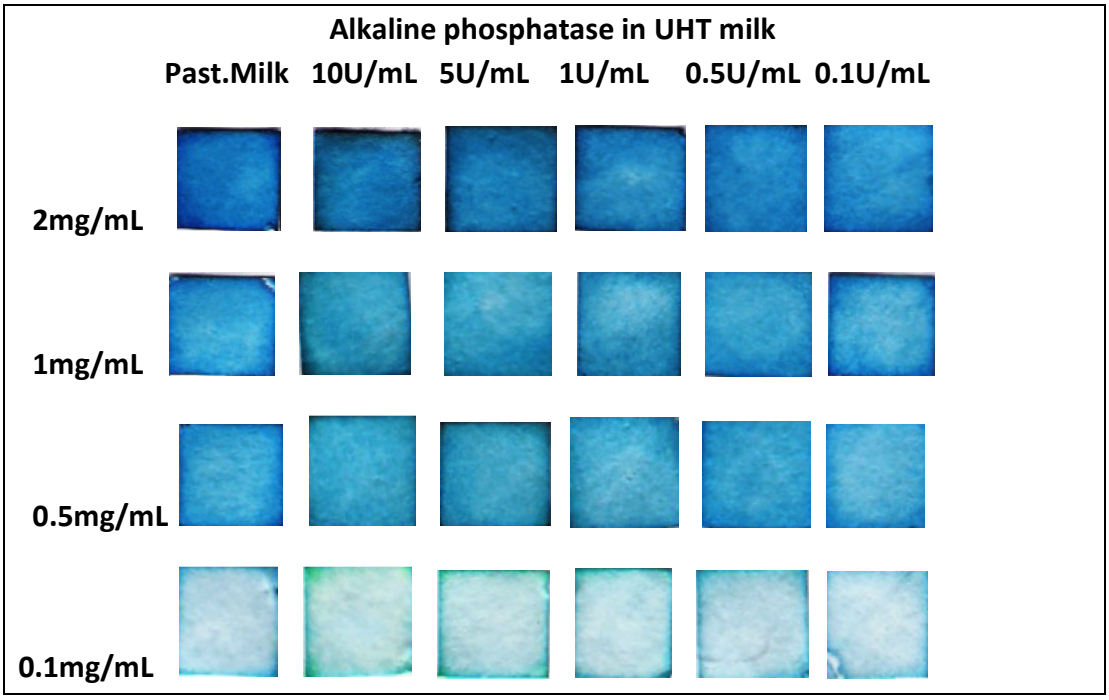

Figure 3.27. The effect of bromocresol green concentration dissolved in 0.1 M glycine buffer at pH 8.5 to the response of bioreporters

The response of papers has been very similar to the papers at pH 8.0. As shown in the figure above increase in the green color intensity with increase in enzyme concentration in milk was not observed at 2 mg/mL and 1 mg/mL of bromocresol green solution at pH 8.5. At these concentration the blue color of bromocresol green solution masked the formation of green color which is results of the enymatic reaction between the alkaline phosphatase and its substrate p-NPP.

According to color intensity analysis (Figure 3.28) the expected relationship between the green color intensity and the enzyme concentration were observed at the papers with 1 mg/mL and 0.5 mg/mL of bromocresol green. However when the color intensity values of papers tested with pasteurized milk compared with milk sample containing different concentration of alkaline phosphatase, approximately

same numerical color values were observed. The bioreporter prepared at this pH value has not differentiated the pasteurized milk and unpasteurized milk. As a results it could be stated that papers at these concentrations of chromogen did not properly work.

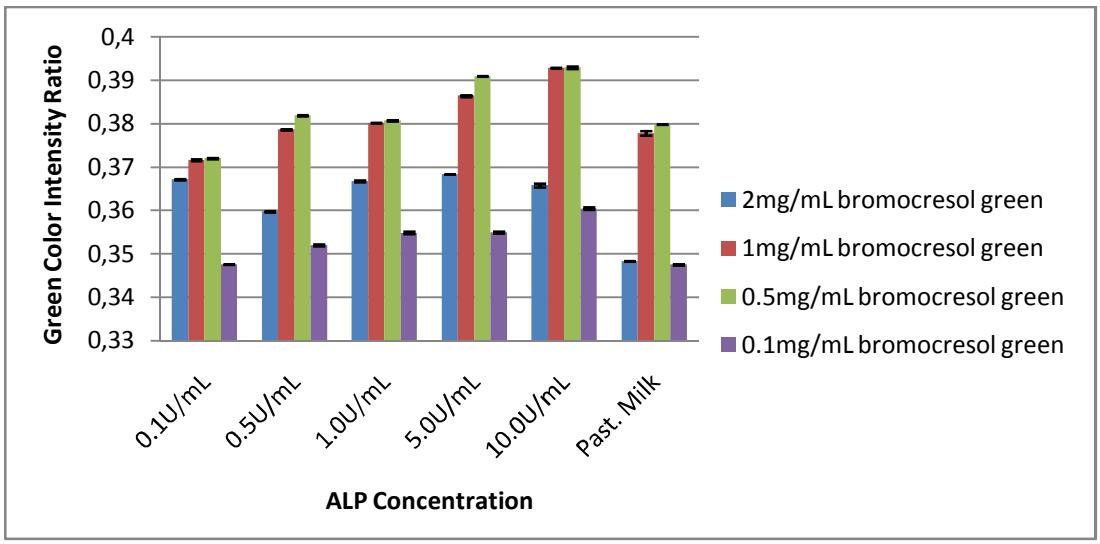

Figure3.28. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 0.1 M glycine buffer at pH 8.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.14 in Appendix B.

In the next experiment glycine buffer at pH 9.0 was used for the preparation of chromogen solution. The responses of these papers (Figure3.29) were almost same as compared with the responses of papers prepared at pH 8.5. The color intensity analysis showed linear increase in green color intensity with increase in enzyme concentration at the papers prepared with 1 mg/mL of bromocresol green (Figure 3.30). However, the responses of papers to different enzyme concentration did not show an efficient differences.

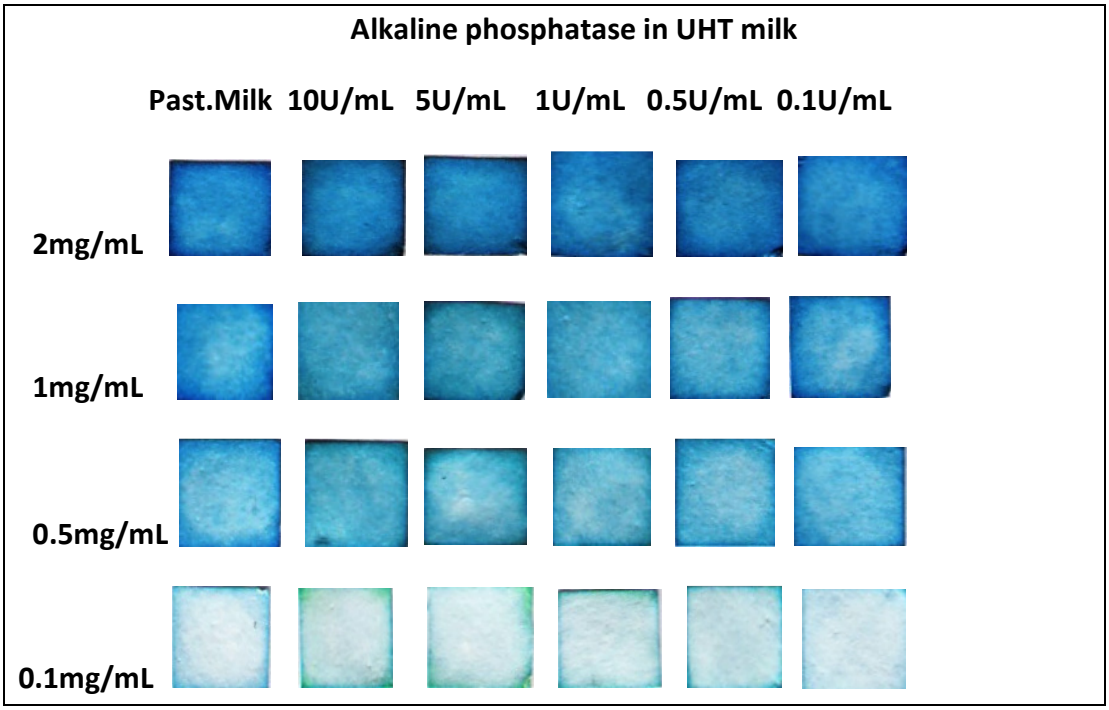

Figure 3.29. The effect of bromocresol green concentration dissolved in 0.1 M glycine buffer at pH 9.0 to the response of bioreporters

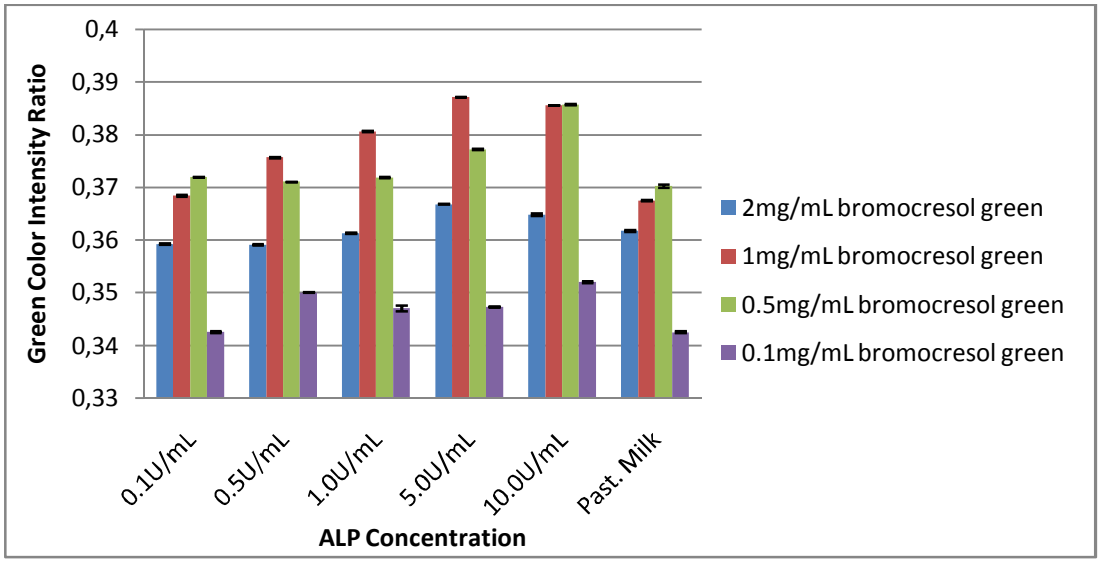

Figure3.30. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 0.1 M glycine buffer at pH 9.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.15 in Appendix B.

Finally, 0.1 M glycine buffer at pH value of 9.5 which was selected as optimum buffer and pH for substrate solution in papers was used to prepare the chromogen solution. As shown in the visual results (Figure 3.31) and the color intensity analysis (Figure 3.32), the increase in the enzyme concentration in milk did not affect the response of papers prepared at each concentration of bromocresol green. As a results of these studies 0.1 M glycine buffer was not selected for chromogen solution in the construction part of bioreporters.

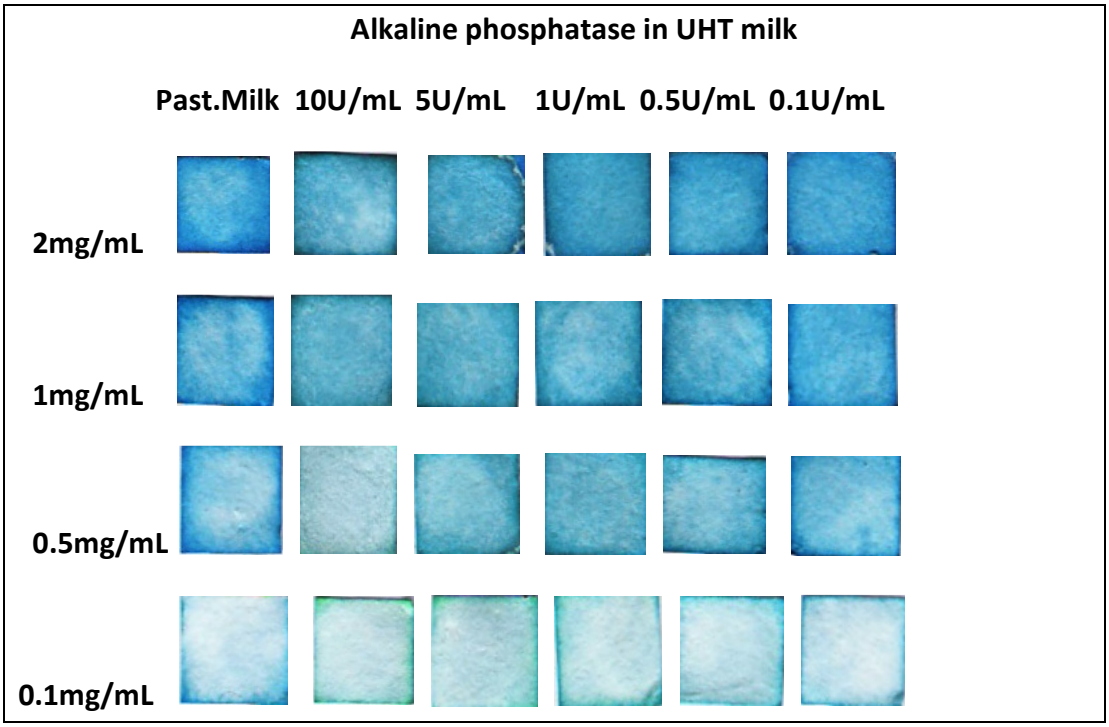

Figure 3.31. The effect of bromocresol green concentration dissolved in 0.1 M glycine buffer at pH 9.5 to the response of bioreporters

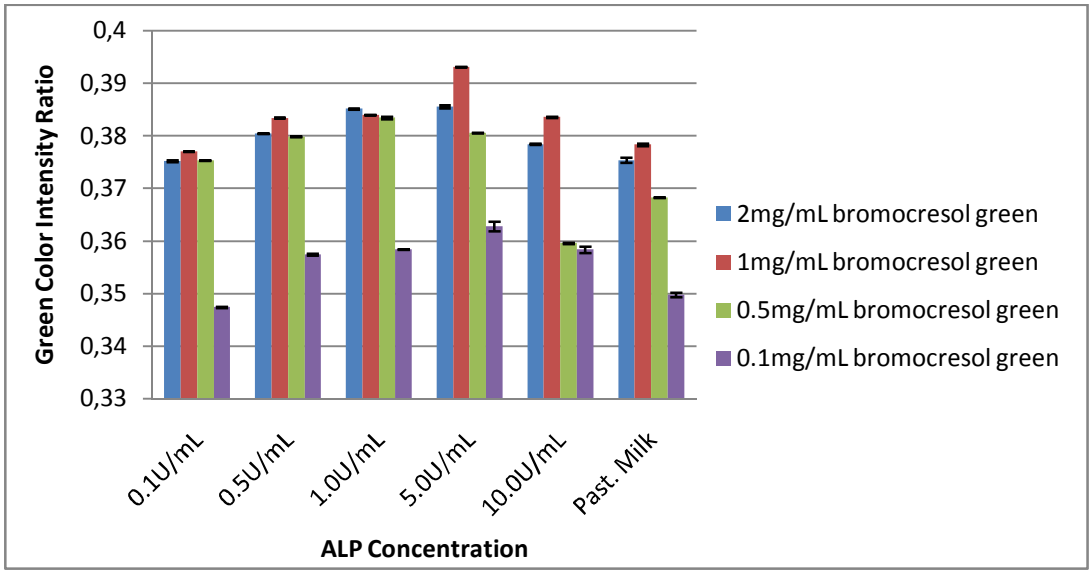

Figure3.32. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 0.1 M glycine buffer at pH 9.5 Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.16 in Appendix B.

3.1.2.2 Bromocresol green in Tris-HCl Buffer

For the optimization studies of chromogen 1.0 M Tris –HCl buffer at four different pH values were also tested and different concentration of bromocresol green solutions were prepared to examine the optimum concentration of chromogen at corresponding pH value of this buffer.

The results of Figure 3.33 belong to the papers prepared with 1.0 M Tris-HCl buffer at pH 8.0.

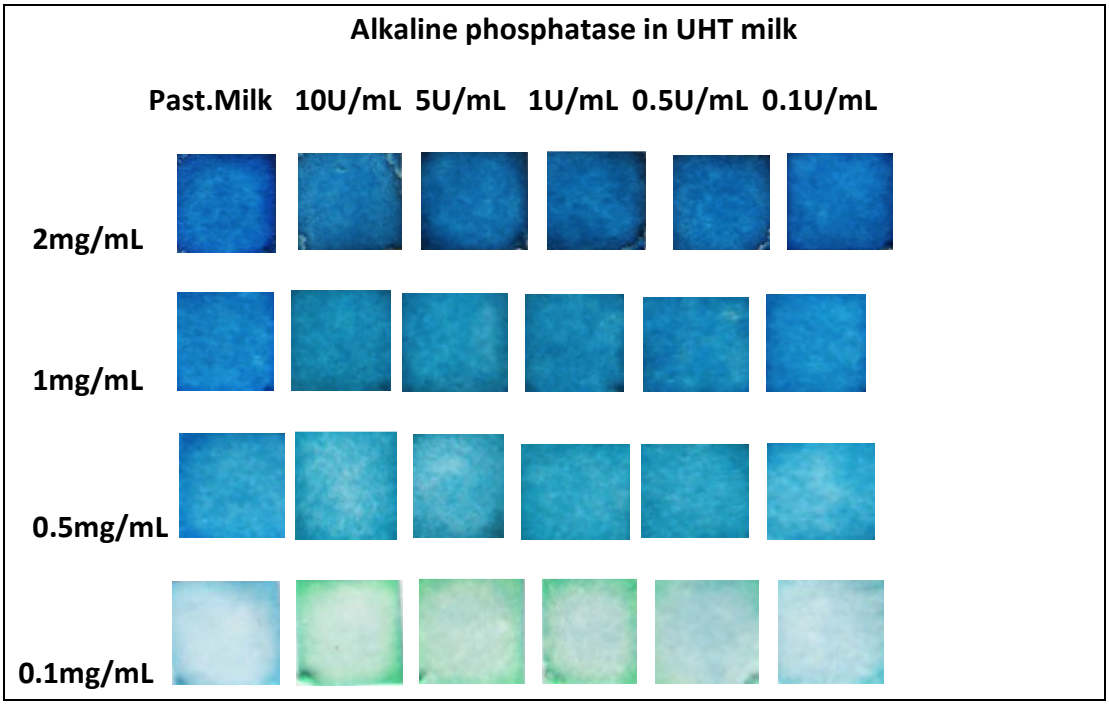

Figure 3.33. The effect of bromocresol green concentration dissolved in 1.0 M Tris-HCl buffer at pH 8.0 to the response of bioreporters

At the bromocresol concentration of 2 mg/mL and 1 mg/mL , the color of the papers were not changing as the enzyme concentration in milk increases (Figure 3.33). This results was also seen in the papers prepared with same concentration of bromocresol green in 0.1 M glycine buffer. The formation of green color at the end of the enymatic reaction between the alkaline phosphatase and its substrate p-NPP was not observed visually due to high concentration of bromocresol green.

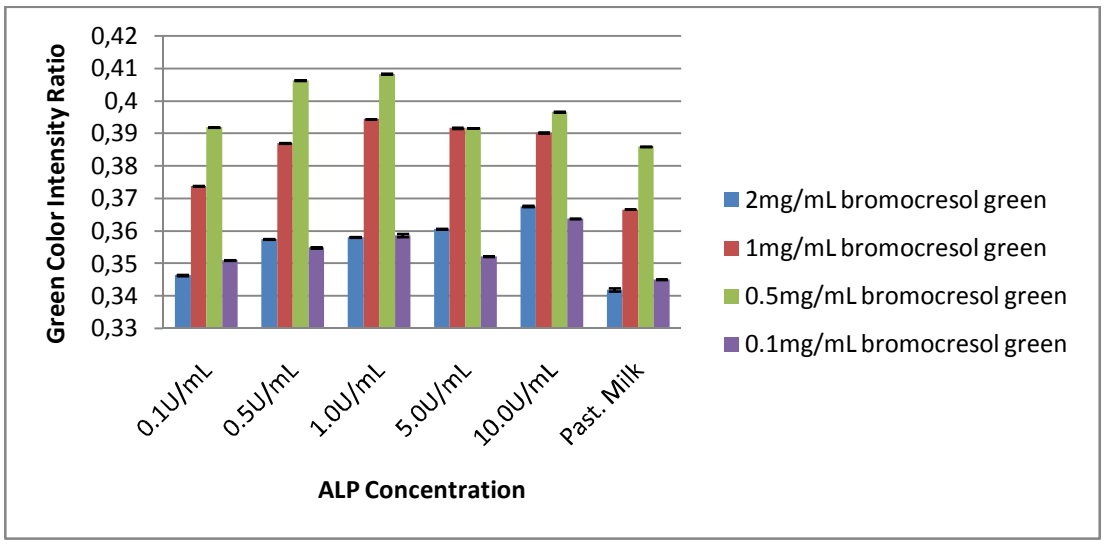

Figure 3.34. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 1.0 M Tris-HCl buffer at pH 8.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.17 in Appendix B.

When the numerical values of color changes were compared (Figure 3.34.) the green color intensitys of papers absorbed with 2 mg/mL of bromocresol green were very low than papers absorped with 1 mg/mL and 0.5 mg/mL. The responses of papers with 2 mg/mL and 0.1 mg/mL were not changing significantly with changing in enzyme concentration. Since the increase in green color intensity of papers with 1 mg/mL and 0.5 mg/mL of bromocresol green was not linear, the chromogen solution prepared at other pH values were studied in the next experiments.

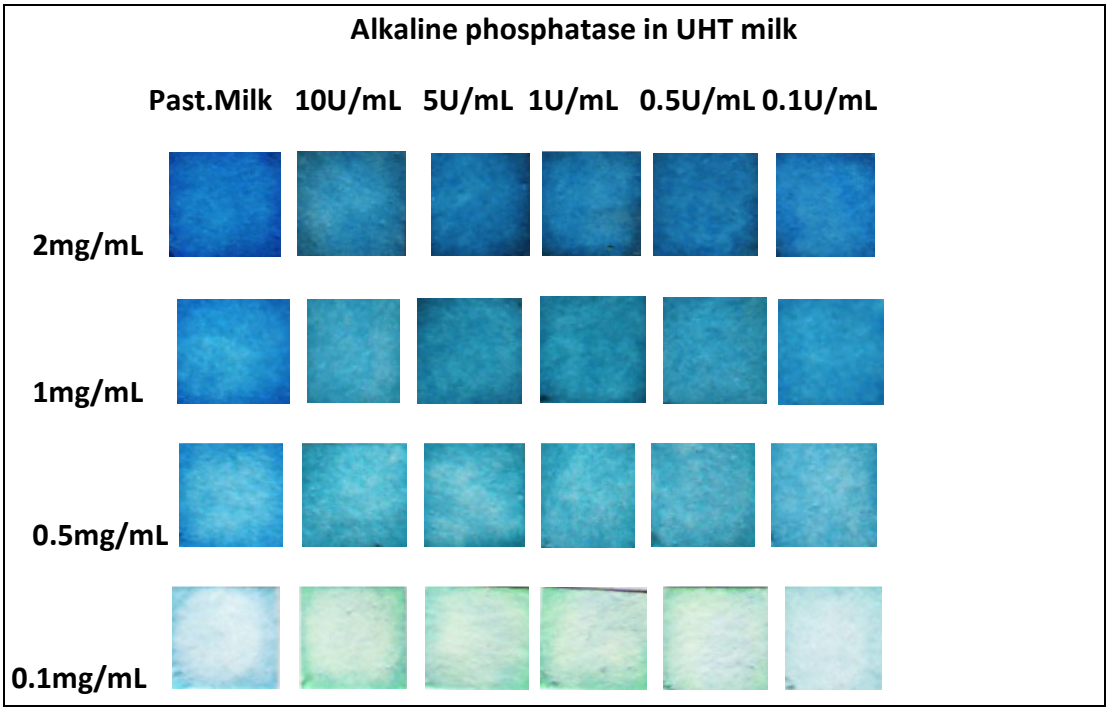

Figure 3.35. The effect of bromocresol green concentration dissolved in 1.0 M Tris-HCl buffer at pH 8.5 to the response of bioreporters

The papers prepared at pH 8.5 showed similar results as shown in figure above. At the high concentration of bromocresol green ,the changes in the enzyme concentration in milk were not reflected in the changes in color of papers.

This result was also concluded in the color intensity anayses of papers at pH 8.5 (Figure 3.36). The green color intensity did not change when alkaline phosphatase concentration increased in milk. The papers prepared at each bromocresol green concentration did not show an efficient differences between the enzyme concentration in milk. Therefore 1.0 M Tris-HCl buffer at pH 8.5 was not selected as optimum buffer of chromogen solution for the construction of bioreporters.

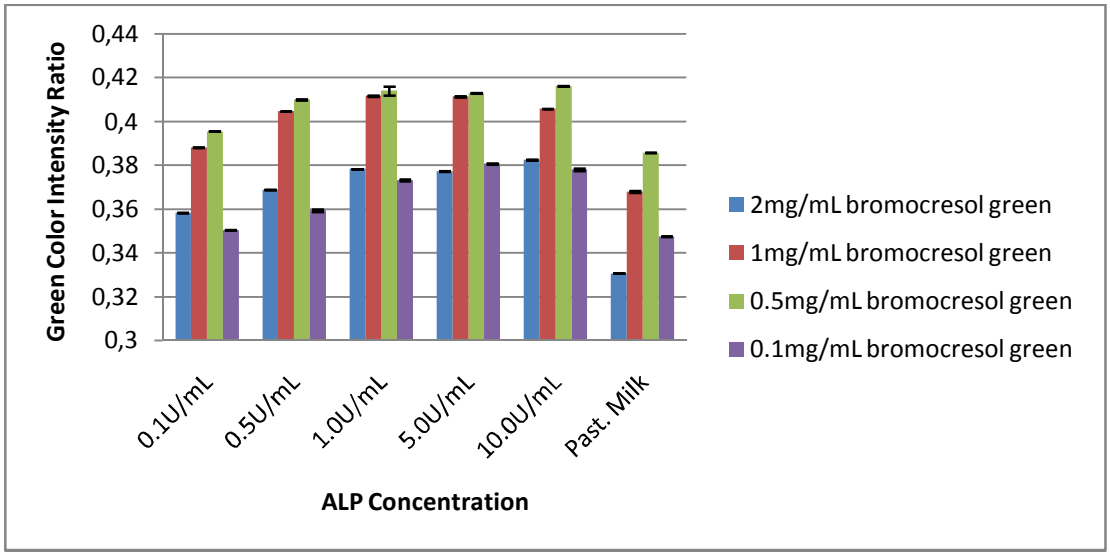

Figure3.36. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 1.0 M Tris-HCl buffer at pH 8.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.18 in Appendix B.

The pH of 1.0 M Tris-HCl buffer was increased to 9.0 for the next experiment. The papers prepared at this pH value were analysed according to the color changes after the reaction between substrate and enzyme on papers. The results were represented in the following figures.

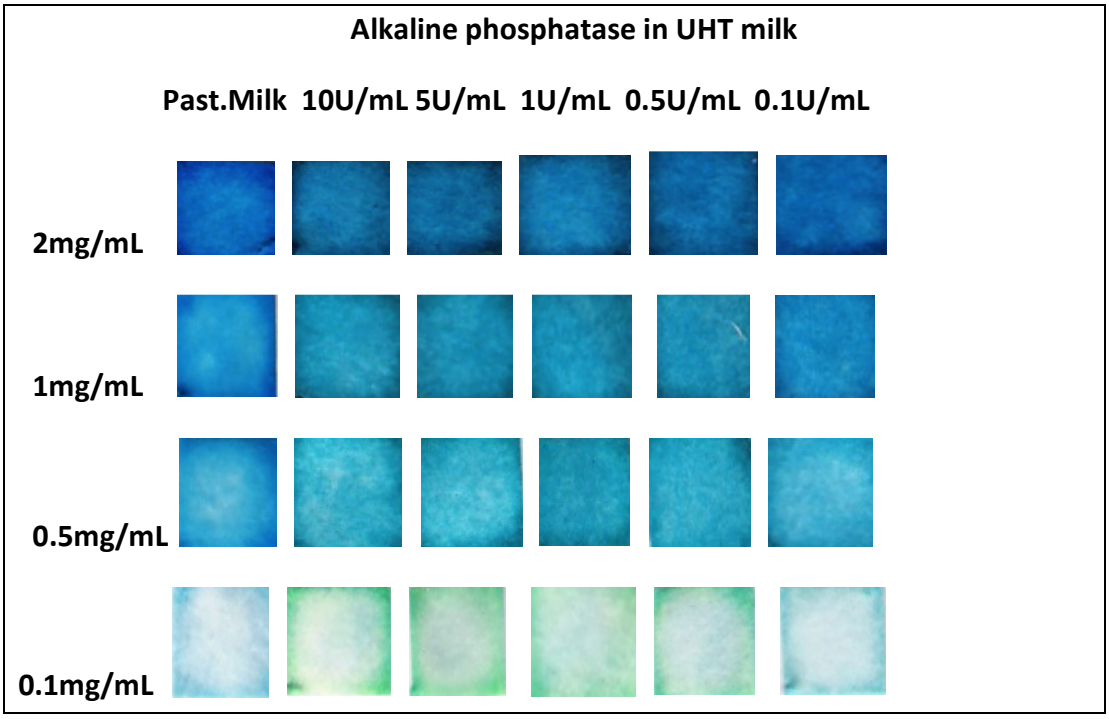

Figure 3.37. The effect of bromocresol green concentration dissolved in 1.0 M Tris-HCl buffer at pH 9.0 to the response of bioreporters

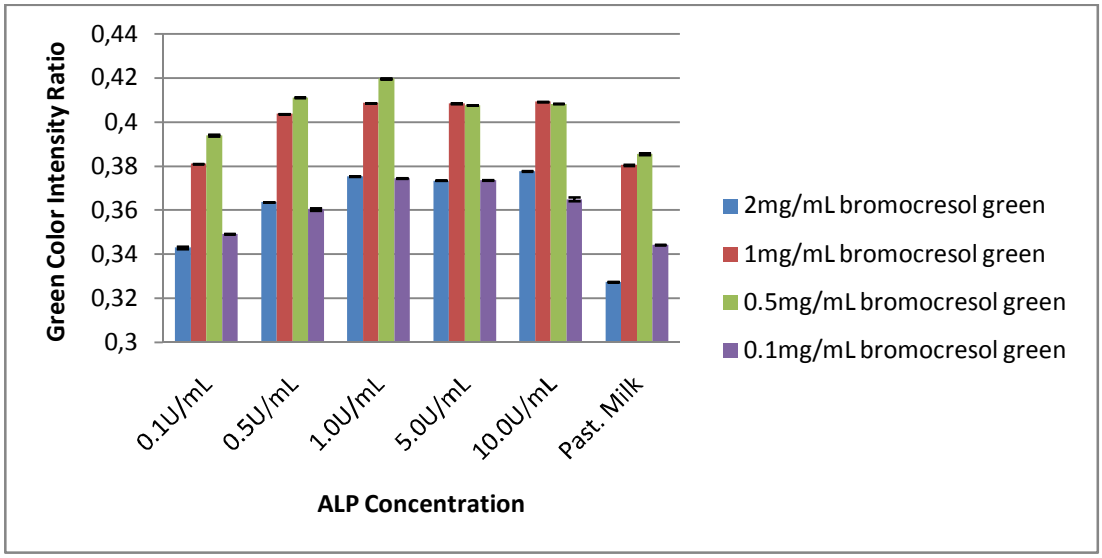

Figure 3.38. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 1.0 M Tris-HCl buffer at pH 9.0. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.19 in Appendix B.

As the response of papers at pH 9.0 to different enzyme concentrations (Figure 3.37) and the numerical analysis of the color intensity value of papers (Figure 3.38) were observed, there was not a significant linear change in the color of papers. the papers prepared at 1.0 M Tris-HCl buffer at pH 9.0 was also regarded as ineffective for the construction of bioreporter to observe alkaline phosphatase concentration in milk.

The last optimization study was performed in 1.0 M Tris-HCl buffer at pH 9.5. Although the responses of papers to different concentration of alkaline phosphatase in milk were observed similar with papers at other pH values (Figure 3.39) , the color intensity analysis (Figure 3.40) showed the expected results. The papers prepared at each concentration of bromocresol green were showed significant increase in green color as the enzyme concentration increased.

Also, the color differences between each intensity of enzyme was obviously observed at each concentration of chromogen. In order to reduce the production cost of bioreporters 0.5 mg/mL of bromocresol green was selected as optimum chromogen concentration although 1 mg/mL and 2 mg/mL of bromocresol green showed effective results.

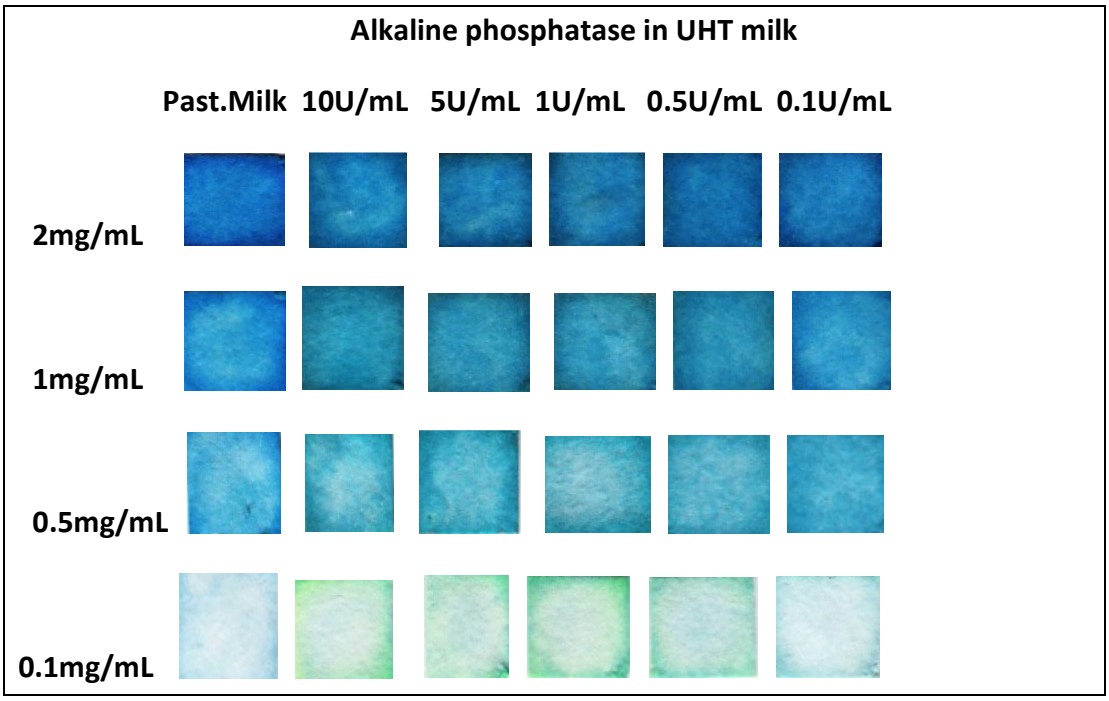

Figure 3.39. The effect of bromocresol green concentration dissolved in 1.0 M Tris-HCl buffer at pH 9.5 to the response of bioreporters

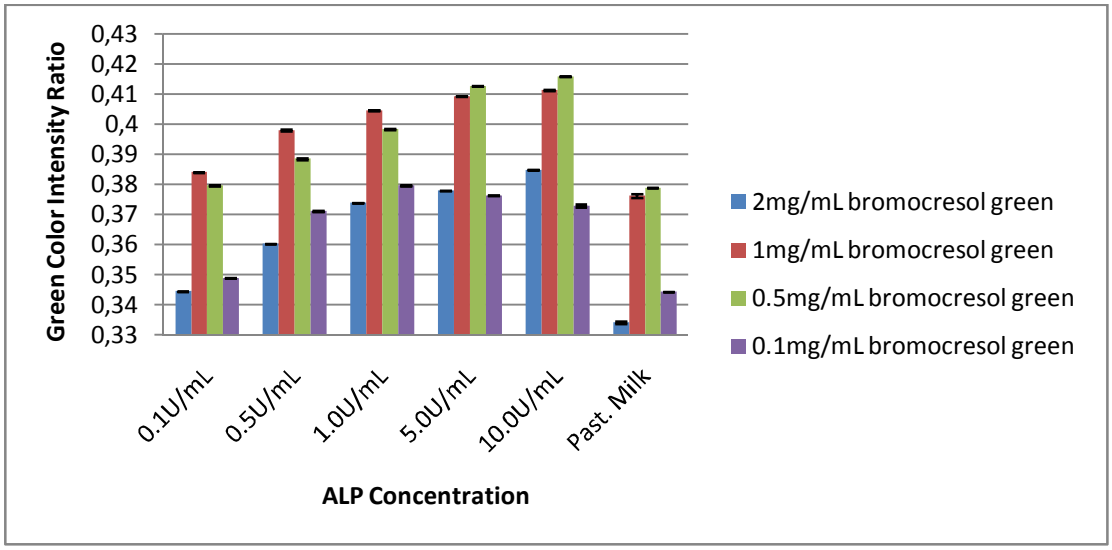

Figure3.40. The analysis of green color intensity ratio of bioreporters prepared with different amounts of bromocresol green in 1.0 M Tris-HCl buffer at pH 9.5. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.20 in Appendix B.

As a result of the studies of chromogen optimization, 0.5 mg/mL of bromocresol green was selected as optimum in Tris-HCl buffer at pH 9.5 for further studies in alkaline phosphatase bioreporter.

3.1.3 Detection limit of Bioreporter

After the optimization studies of substrate and chromogen the detection limit of bioreporter for alkaline phosphatase in milk was studied. The papers were prepared with optimum concentration of p-NPP and bromocresol green which were 1 mg/mL in 0.1 M glycine buffer at pH9.5 and 0.5 mg/mL in 1.0 M Tris-HCl buffer at pH 9.5 respectively. The milk samples containing a range of alkaline phosphatase concentration were prepared in UHT milk. After the absorption of p-NPP-bromocresol mixture to papers and drying in vacuum dessicator milk samples were applied to the papers. The formation of green color in papers were observed

and color intensity analysis was performed to have numerical values in the color changes. As a control 0.1 M glycine buffer at pH 9.5 and UHT milk sample were also applied to papers to compare the concentration of alkaline phosphatase in milk.

The following results in Figure 3.41 show the response of papers to alkaline phosphatase concentration in milk.

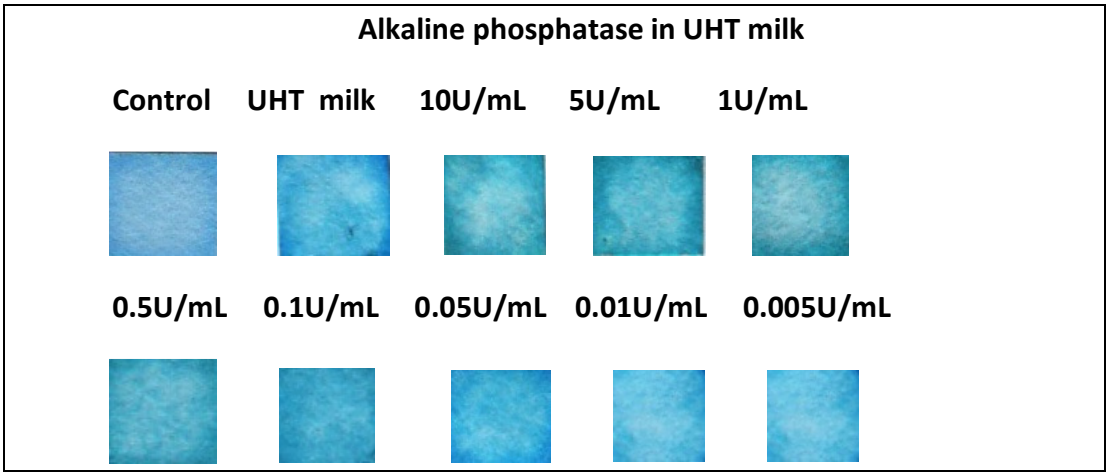

Figure 3.41 The detection limit of alkaline phosphatase in milk.

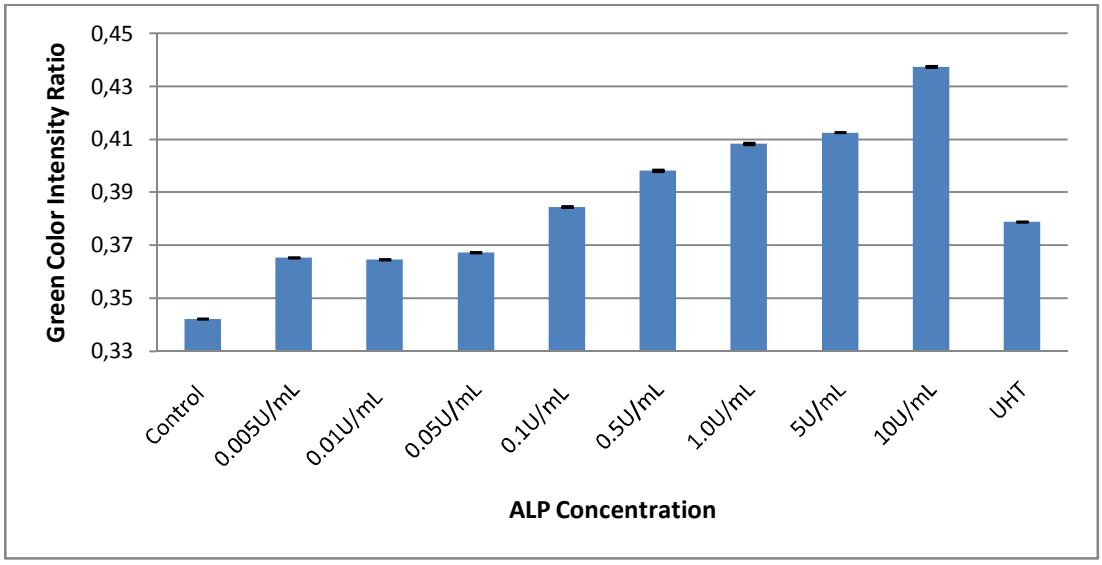

Figure 3.42.The analysis of green color intensity ratio for the detection limit of alkaline phosphatase in milk. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.21 in Appendix B.

According to the color change in papers after the applicaiton of milk samples (Figure3.41) , it can be stated that the detection limit of alkaline phosphatase was 0.5 U/mL. The formation of green color was easily observed at 0.5 U/mL and higher concentration of alkaline phosphatase in milk.

Although visually the detection limit of alkaline phospahatase was determined as 0.5 U/mL , the color intensity analysis (Figure 3.42) showed that the green color intensity of 0.1 U/mL and higher alkaline phosphatase concentration were different than milk samples of 0.05 U/mL and lower concentrations.

The Turkish Standard Instituton states the limit of alkaline phosphatase in pasteurized milk as at most 0.5 U/mL (Turkish Standard, TS 1019, 2002). The milk containing higher concentration of alkaline phosphatase is regarded as

unpasteurized milk. Our aim is to constract a bioreporter which reports the presense of alkaline phosphatase in milk samples semiquantitatively. Therefore, the detection limit of 0.5 U/mL of alkaline phosphatase according to the visual analysis makes our bioreporter applicaple for the control of effiency of pasteurization process.

3.2 Effect of Sample Temperature on the Response of Bioreporter

For the construction of bioreporter optimization studies were performed with milk samples kept at room temperature before their application to papers. However, in order to produce an efficient bioreporter for the detection of alkaline phosphatase the effect of temperature of milk samples were studied. The papers were prepared according to the optimization studies. The milk samples were stored at 4˚C, room temperature and 37˚C for one hour before applied to papers.

As shown in the Figure 3.43, visual analysis showed that the temperature of milk samples do not affect the response of bioreporters. The color difference between the milk samples at 0.5 U/mL and pasteurized milk can easily observed at each temperature.

According to the color intensity analysis represented in Figure 3.44, the green color formation on the paper was observed at higher numerical value after the application of milk sample at 37˚C.

Since the response of papers which means the formation of green color on papers applied with unpasteurized milk is not affected from the sample temperature, all milk samples can be tested with our bioreporter.

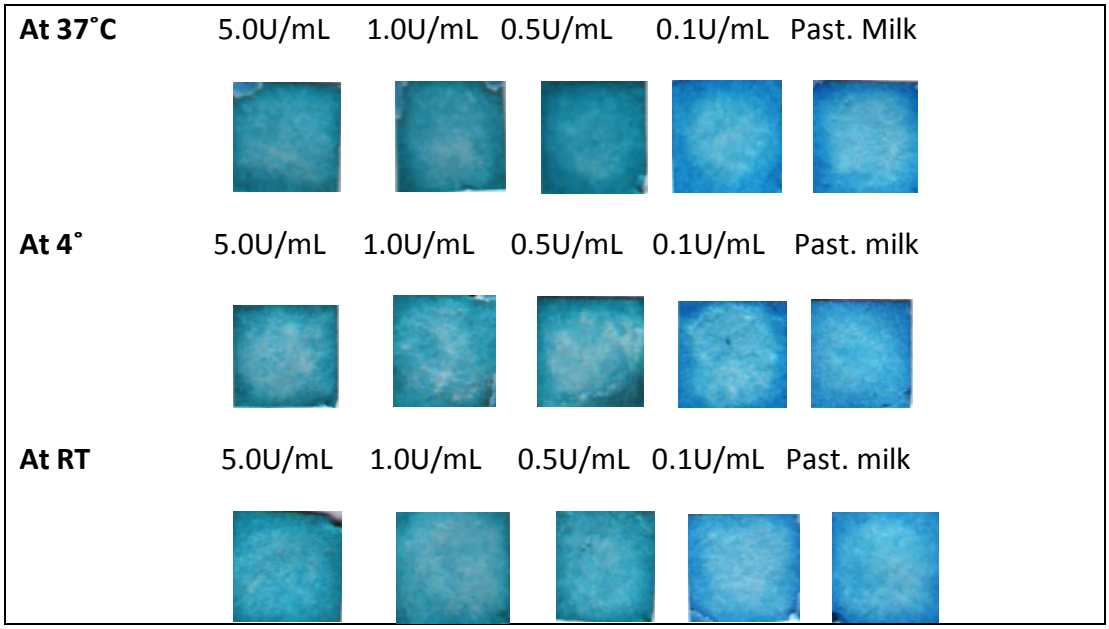

Figure 3.43.The effect of sample temperature to the response of bioreporter.

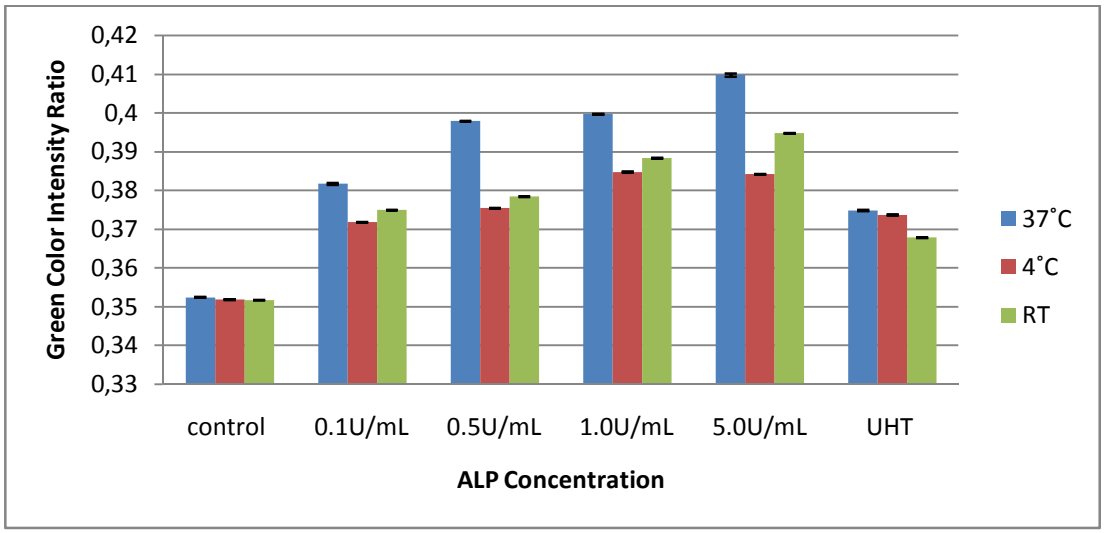

Figure 3.44.The analysis of green color intensity ratio of bioreporters to milk samples stored at different temperature. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.22 in Appendix B.

3.3 Effect of Sample pH on the Response of Bioreporter

In addition to the effect of sample temperature, pH of milk samples were also evaluated for their effect on the response of bioreporters. The pH of milk samples at concentration of 0.1 U/mL, 0.5 U/mL and 1.0 U/mL of alkaline phosphatase and UHT milk were adjusted within a range of 5.0-6.5 and applied to papers prepared in optimum condition. The lower pH values were not adjusted because of the precipitation of milk proteins at lower pH values. After the application of milk samples the color changes on papers were analysis both visually and numerically based on the color intensitys.

The enzymatic reaction between the substrate, p-NPP, and alkaline phosphatase has optimum activity at alkaline pH due to being optimum pH condition for enzyme. However, the pH value of raw milk is generally 6.6 due to the complex structure of milk with different proteins, natural enzymes and fat molecules (Wilin´ska et al., 2007). Therefore, in this study the pH range of 5.0 to 6.5 were studied.

According to the results of analysis represented in Figure 3.45 and Figure 3.46 the same conclusion was brought out from the papers. The pH of samples does not results any change in the response of bioreporter. Our bioreporter can be applicable for all milk samples within a pH range of 5.0 to 6.5. Moreover, as the pH value of milk samples increases the color intensity observed on papers represents higher green color values. This results state that as the pH value of milk sample approaches to the optimum pH of alkaline phosphatase, greater activity can be observed.

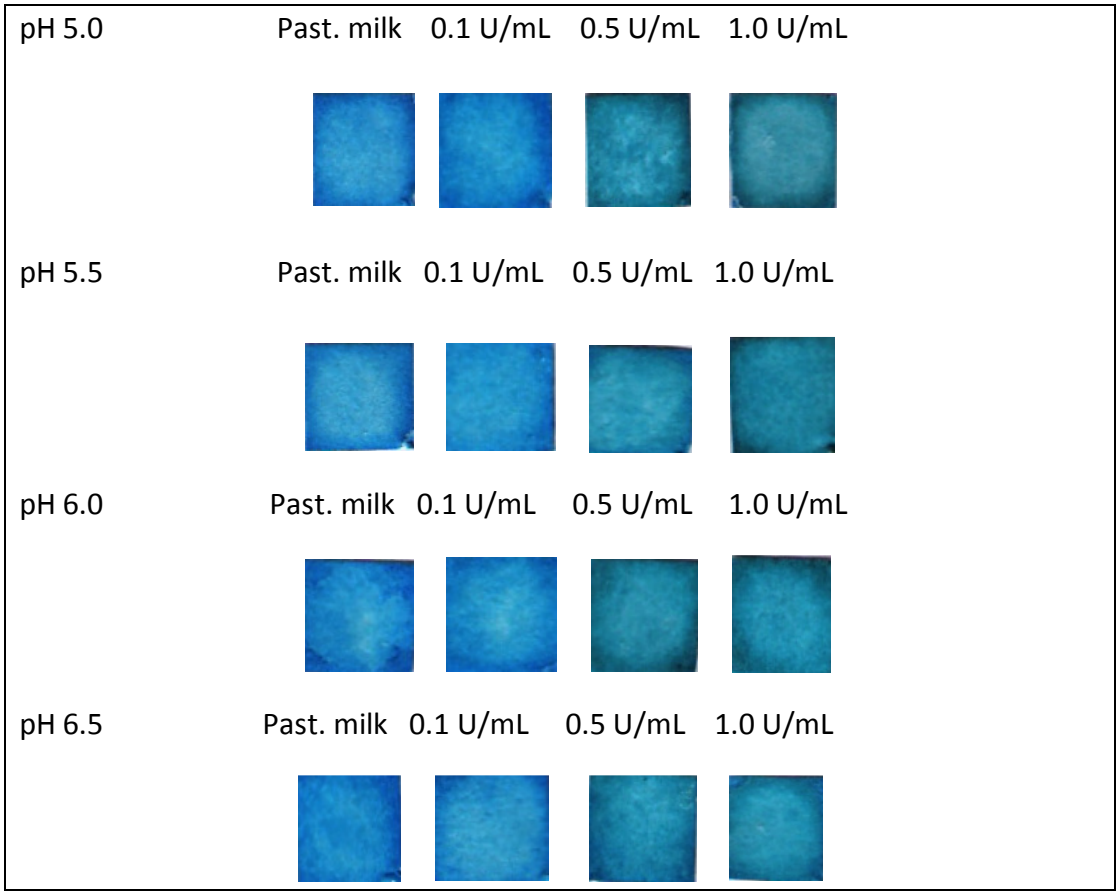

Figure 3.45. The effect of sample pH to the response of bioreporters

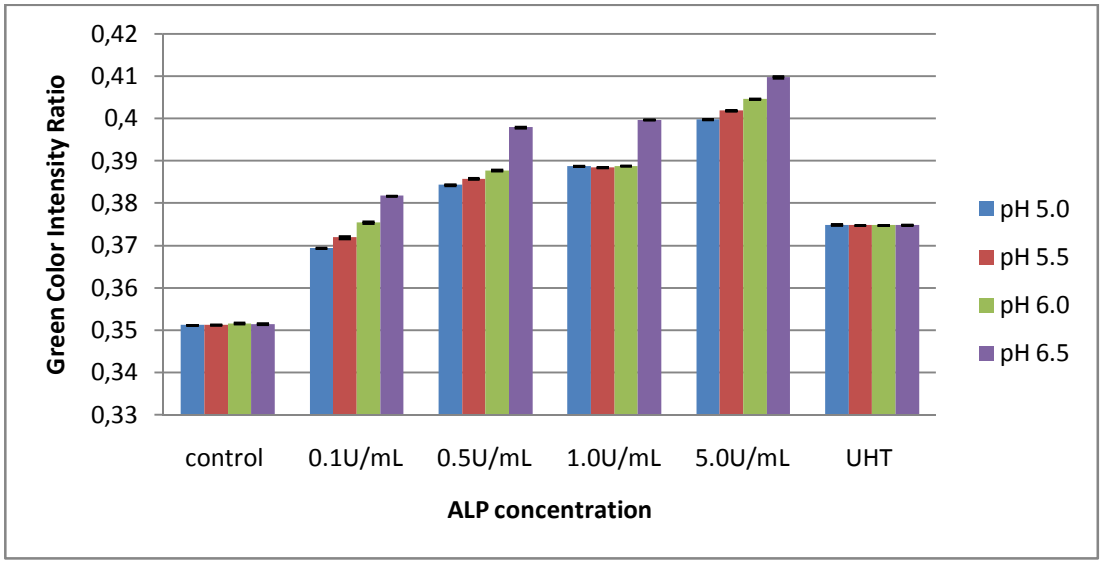

Figure 3.46.The analysis of green color intensity ratio of bioreporters to milk samples at different pH. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.23 in Appendix B.

3.4 The Response of Bioreporter to Different Milk Samples

Alkaline phosphatase is a natural raw milk enzyme which denatures at the pasteurization process of milk. Based on its higher resistance to thermal processes than pathogenic microorganisms in raw milk, alkaline phosphatase is used as reporter for the efficiency of pasteurization process (Wilin'ska *et al*., 2007).

The activity of alkaline phosphatase in milk has shown differences among the source of milk (from which animal), the physiological conditions during lactation period of animal, the seasonal change, the location and some other factors (Fox & Kelly, 2006).

In this study, our aim is to construct a bioreporter for the control of pasteurization process based on the level of alkaline phosphatase in every kinds of milk required to be tested. Therefore, in order to ensure the appropriateness of bioreporters to

different milk samples in terms of their source and location the response of bioreporters were studied with constructed alkaline phosphatase bioreporter. As a control glycine buffer was applied to papers to observe any negative results on papers.

3.4.1 Milk Samples from Different Animal Sources

In this study the bioreporters were tested with milk samples from different animal sources. The milks of cattle, goat and sheep were compared according to their color formation on papers prepared with optimum substrate and chromogen solution. In order to be sure that any solutions except one containing alkaline phosphatase does not cause any color change on papers, UHT milk and glycine buffer were also tested.

In the following figure, it has been shown that only milk samples from cattle and sheep show the response as forming clear green color on papers.

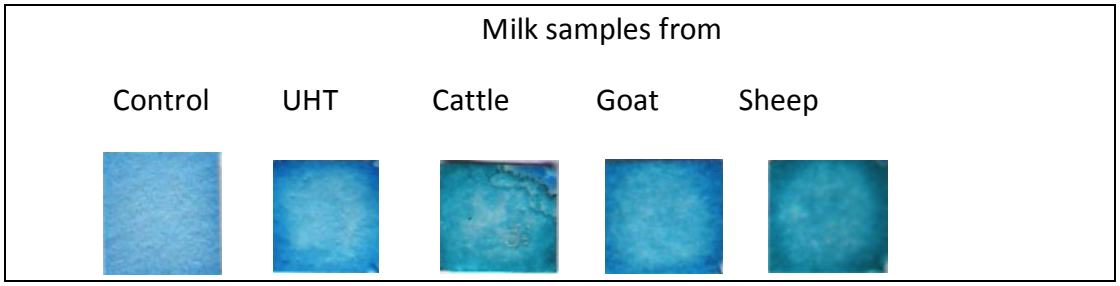

Figure 3.47. The effect of milk samples from different source of animal to the response of bioreporters

According to the numerical value of papers represented in Figure 3.48, higher alkaline phosphatase activity has been observed in milk samples from cattle and

sheep. In the study of Ljutovac *et al.,* it has been stated that the activity of alkaline phosphatase in cow milk is higher than the activity in goat milk (Ljutavac et al., 2007).

In our study, the detection limit was determined as 0.5 U/mL of alkaline phosphatase according to the Turkish Standard Institution (TS 1019, 2002). Therefore, this bioreporter can report the presence of 0.5 U/mL and higher values of alkaline phosphatase activity in milk. The goat milk samples having at least 0.5 U/mL of alkaline phosphatase activity has shown responses otherwise this bioreporter can not be used for the control of pasteurization process in goat milk.

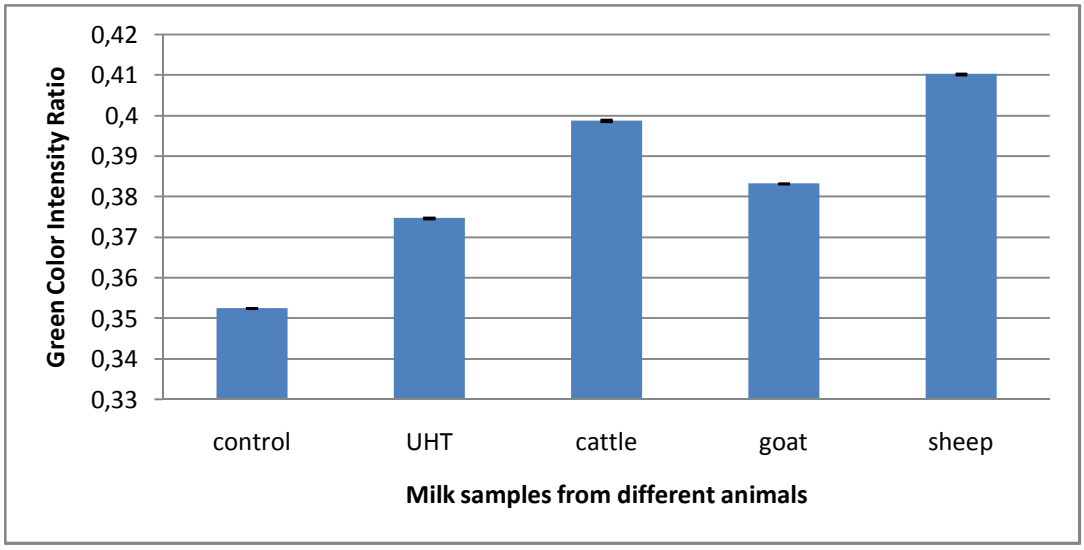

Figure3.48 The analysis of green color intensity ratio of bioreporters to milk samples from different animal source. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.24 in Appendix B.

3.4.2 Lipid Free Milk Samples

The enzyme activity in milk has not been analysed practically because of the components of milk which make it complex (Dinnella *et al.*, 2004).The presence of certain component can cause failure in the measurement of enzyme activity or even results in the denaturation of enzymes in milk (Dinnella *et al.*, 2004).

In this study , our aim is to determine the effect of lipid molecules to the activity of alkaline phosphatase in milk samples. The lipid of raw milk samples were removed by centrifugational force and the resulting lipid free milk was applied to constructed papers.

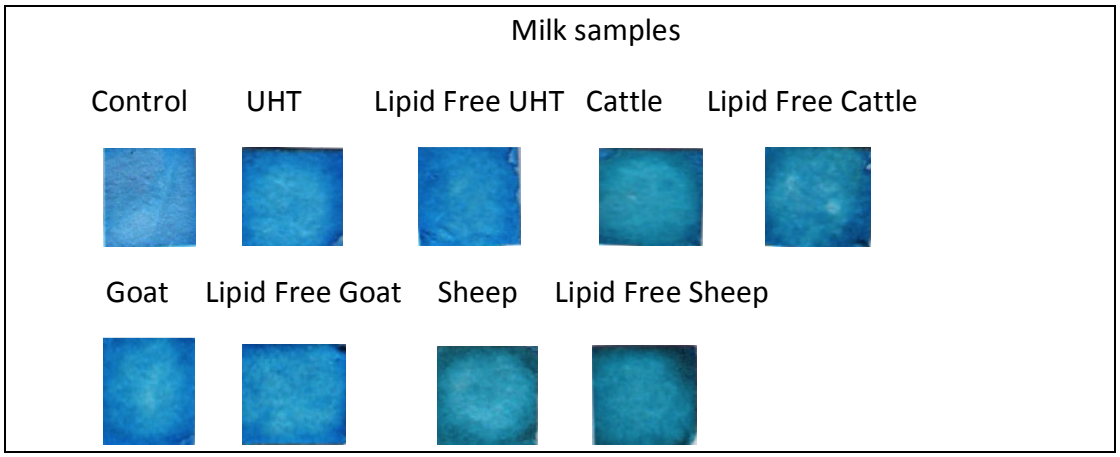

Figure 3.49. The effect of lipid in milk samples to the response of bioreporters

The response of bioreporter has not been affected with the removal of lipid from milk samples, as shown in Figure 3.49. This result has been also supported by the color intensity analysis (Figure 3.50). As a result, it can be concluded that the alkaline phosphatase bioreporter can be used also for lipid free raw milk .

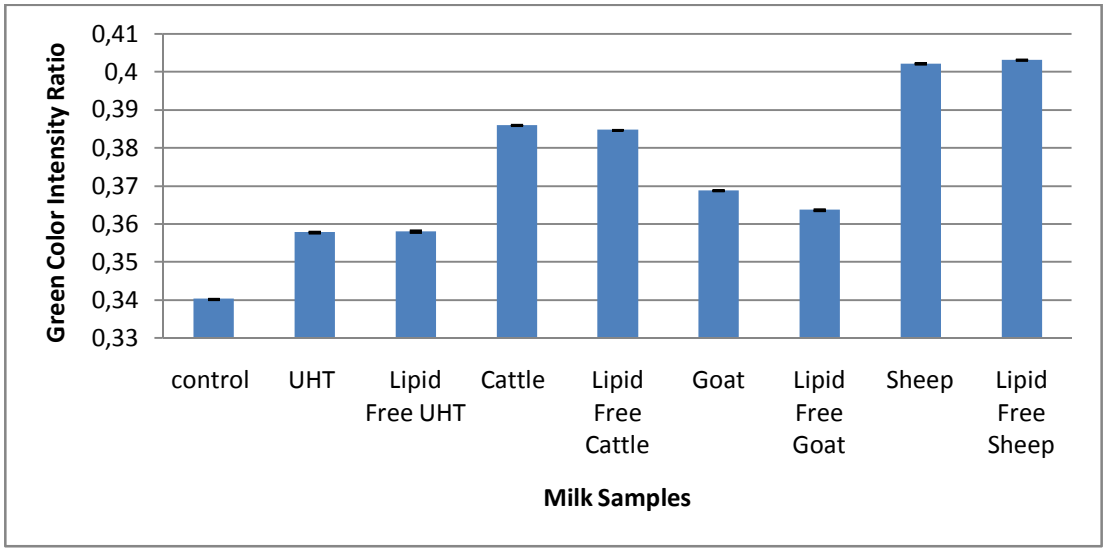

Figure 3.50. The analysis of green color intensity ratio of bioreporters to lipid free milk samples from different animal source. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.25 in Appendix B.

3.4.3 Milk Samples from Different Location in Turkey

Beside milk samples from different animal source, location of milk source was also evaluated based on their effect on the response of bioreporter. The milk samples used in this study came from different regions of Turkey.

As the response of papers has been shown in Figure 3.51, the activity of alkaline phosphatase have shown different results as the location of milk source has been changing in Turkey. This difference in activity has been also represented in the numerical analysis of the color of papers (Figure 3.52). The green color on the papers tested with raw milk from different region have been obvious as compared with UHT milk.

These results have provided the conclusion that our bioreporter has a wide range of application area due to its positive responses to different source and different location of milk.

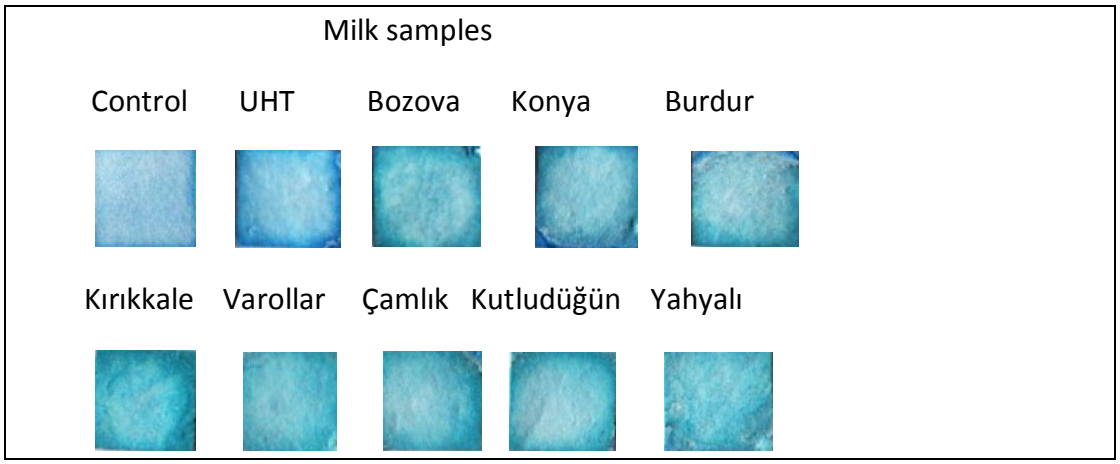

Figure 3.51. The effect of milk samples from different location of Turkey to the response of bioreporters

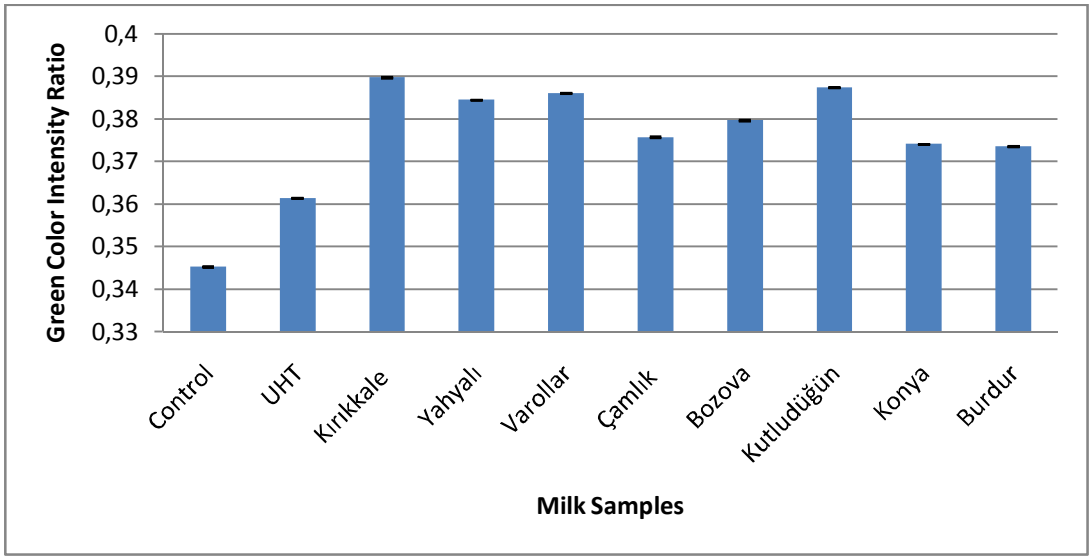

Figure3.52.The analysis of green color intensity ratio of bioreporters to milk samples from different location in Turkey. Error bars represent standart error of mean (SEM).The tabulated intensity ratio values of graph are provided at Table B.26 in Appendix B.

3.5 The Application of Heat Treatment to Milk Samples

In food industry the thermal processes play important roles for the safety of products. One of the thermal processes in food industry, pasteurization process, provides the dairy product with safety quality and increasing in the shelf life (Dinnella *et al.*, 2004).

In order to increase the shelf life of end product and provide the safety quality the application of heat in process has to provide the inactivation of microorganisms without affecting the nutritional value of milk. For this purpose, time-temperature combination is evaluated as most significant parameter in pasteurization process (Mortier *et al.,* 2000).

According to the Turkish Standard Institution, the effective time-temperature combination is stated as 63˚C for 30 min or 72˚C for 15 sec for proper pasteurization process (TS 1019, 2002).

In our bioreporter, the aim is to control the pasteurization process of milk by reporting the presence of alkaline phosphatase in milk with color change on papers. In this part of study, we applied heat treatment at different temperature to milk samples with known concentration of enzyme and raw milk samples from different location of Turkey. The responses of papers were evaluated by color intensity analysis.

In the first set of experiment the milk samples were applied a heat treatment at 50˚C for 30 min. At 10 min intervals the milk samples were taken and applied to papers in order to observe the response of bioreporter. Since the temperature applied to milk samples was lower than the temperature applied at pasteurization process, it has been expected that at the end of the treatment the activity of alkaline phosphatase is still observed.

As Figure 3.53 has shown, the color intensitys of papers have been decreasing from the beginning of treatment towards to the end at each concentration of alkaline phosphatase in milk. However, at the end of heat treatment milk samples containing 0.5 U/mL and higher enzyme activity have still shown higher green color value than pasteurized milk (purple bars in Figure 3.53). These milk samples have been still unpasteurized after the application of heat treatment at 50˚C for 30min.

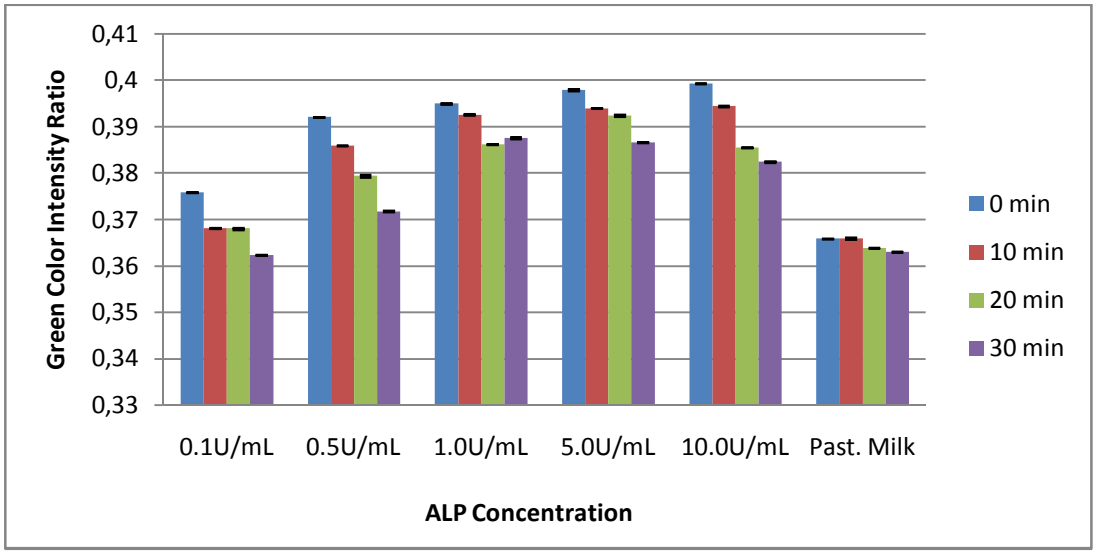

Figure 3.53.The analysis of green color intensity ratio of bioreporters to milk samples treated at 50˚C for 30 min. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.27 in Appendix B.

After proving that our bioreporter shows proper responses to the milk samples treated at lower temperature than pasteurization process, next experiment was performed with milk samples treated at 60˚C for 30min. At 10 min intervals the milk samples were applied to papers.

The Figure 3.54 represents the color intensity analysis of this study. Before the application of heat treatment the color intensity values of milk samples have been very high and shown increasing value as the enzyme concentration has been increasing in milk samples (Blue bars in Figure 3.54). However, at the end of the heat treatment at 60˚C these values have decreased significantly (purple bars). When the differences in the color intensity of milk samples containing alkaline phosphatase concentration at least 0.5 U/mL and pasteurized milk have been compared, it can be concluded that these milks have been still unpasteurized.

However, the activity of enzyme has decreased at heat treatment 60˚C significantly.

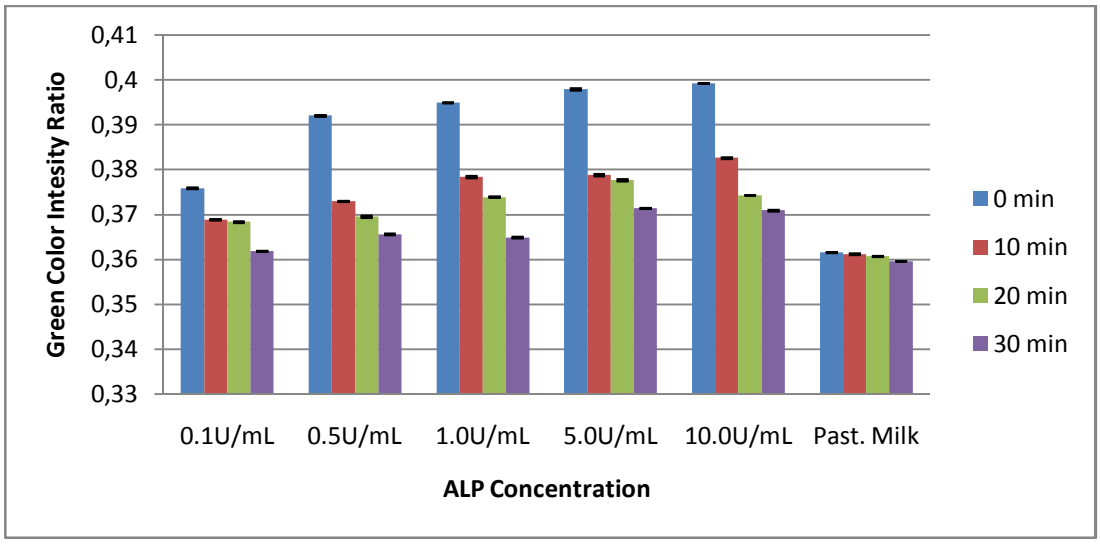

Figure 3.54. The analysis of green color intensity ratio of bioreporters to milk samples treated at 60˚C for 30 min. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.28 in Appendix B.

In addition to the results obtained from milk samples with known amount of alkaline phosphatase, our bioreporter was also tested with raw milk samples taken from different location of Turkey. The milk samples were heated at 50˚C and 60 ˚C for 30 min and the formation of green color on papers were analysis in color intensity analysis.

The following figures have represented the color analysis of heat treated milk samples at 50˚C and 60 ˚C respectively. These results have been similar to the

results of milk samples with known amount of alkaline phosphatase represented in Figure 3.55 and 3.56.

After the application of heat treatmet at 50 ˚C for 30 min, raw milk samples from different region have been still unpasteurized eventhough the activity of alkaline phosphatase has decreased. The color intensity values of milk samples after 30 min are higher as compared with pasteurized milk (Figure3.55).

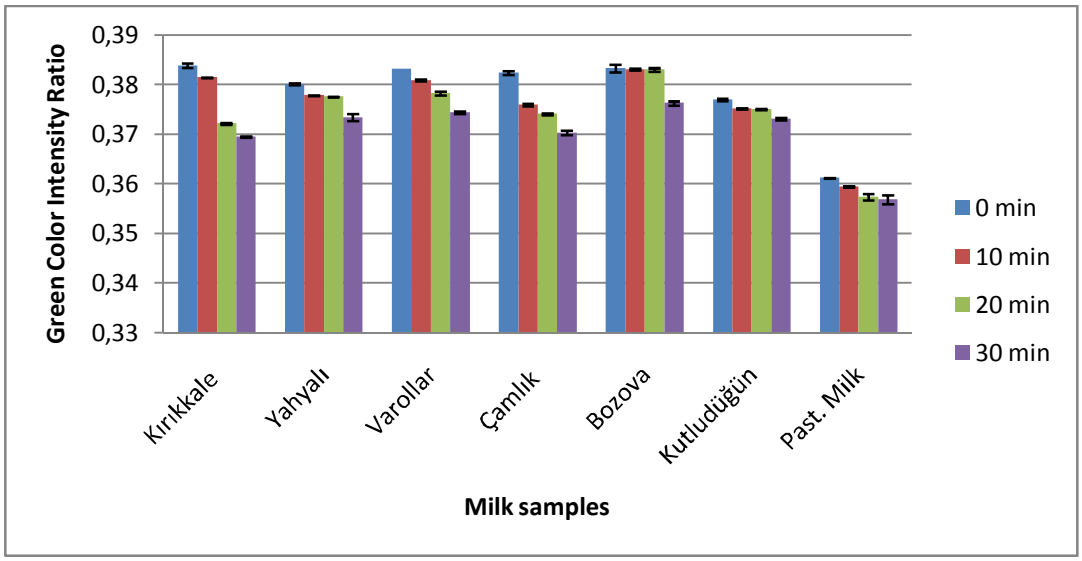

Figure 3.55. The analysis of green color intensity ratio of bioreporters to raw milk samples from different locations in Turkey treated at 50˚C for 30 min. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.29 in Appendix B.

As the heat treatment was performed at 60˚C, the decrease in the activity of alkaline phosphatase was higher in each milk samples (Figure 3.56) since temperature applied to milk samples was close to the pasteurization temperature.

At the end of treatment the milk samples have not been pasteurized properly and this results has been provided with the responses of our bioreporter.

The results taken from the heat treatment studies proved the applicability of our bioreporter in the control of proper pasteurization of the milk.

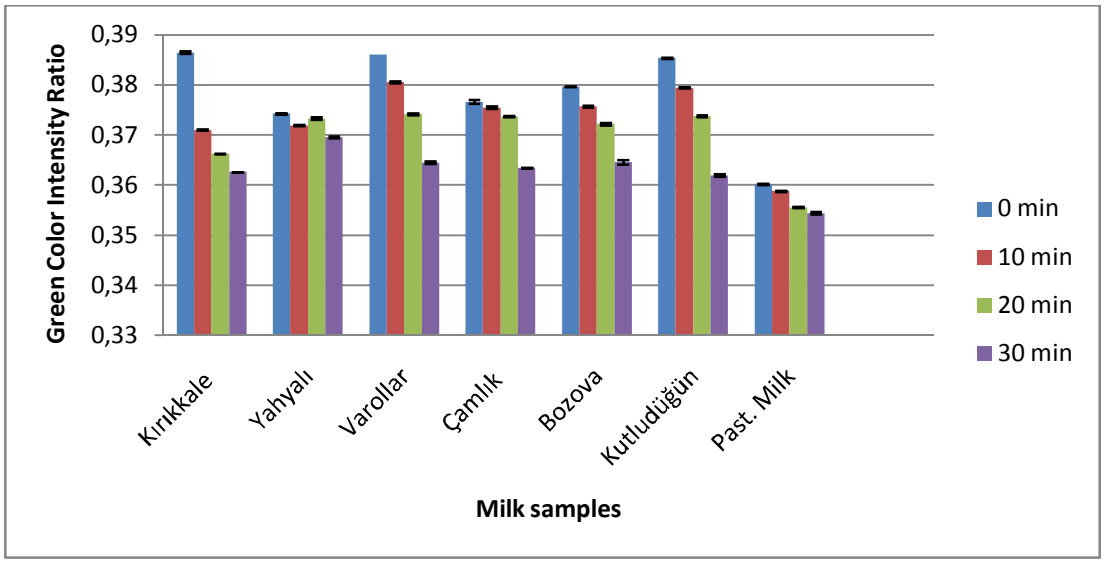

Figure3.56. The analysis of green color intensity ratio of bioreporters to raw milk samples from different locations in Turkey treated at 60˚C for 30 min. Error bars represent standart error of mean (SEM). The tabulated intensity ratio values of graph are provided at Table B.30 in Appendix B.

3.6 The Addition of Raw Milk to Pasteurized Milk

Since the thermal processes denature alkaline phosphatase found naturally in raw milk, the presence of it in pasteurized milk indicates the ineffective pasteurization process or the contamination of end product with raw milk in the system (Dinnella *et al.,* 2004).

In this study the response of the bioreporter to contaminated milk samples were evaluated. Pasteurized milk contaminated with raw milk in a range of 1 to 90% was applied to papers and the detection limit of contamination were determined according to the analysis.

Figure 3.57 shows the color change on papers after the application of contaminated milk samples. As a control pasteurized milk was also applied to paper.

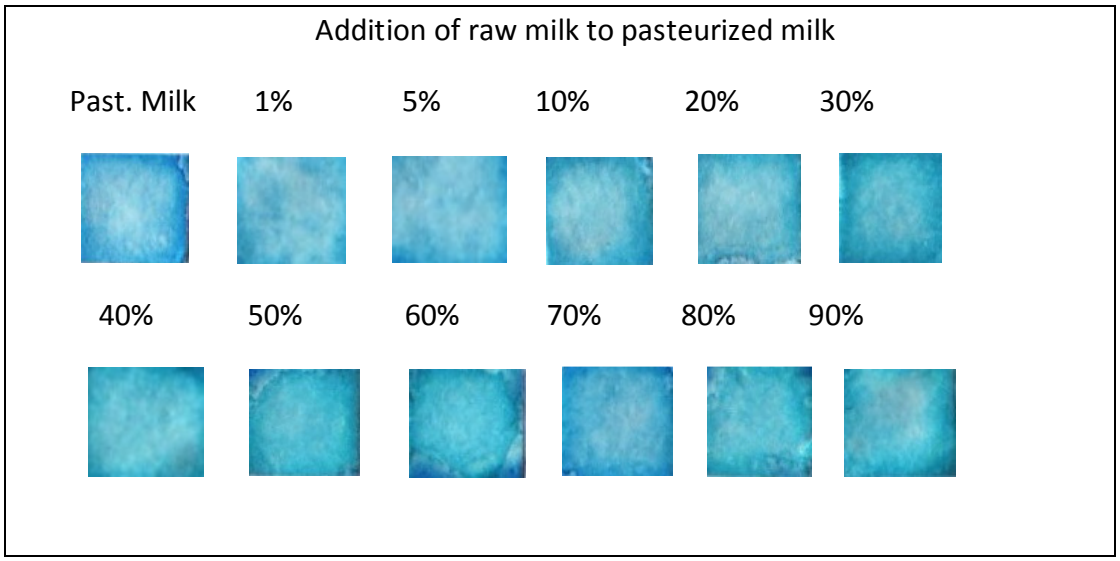

Figure 3.57. The mixing of pasteurized milk with raw milk sample from Kırıkkale. The percentages represent the percentages of raw milk added to pasteurized milk.

While there is a clear color change observed on papers above 10% contamination (Figure 3.57), with green color intensity ratio analysis , contamination as lower as 1% can be detected (Figure3.58).

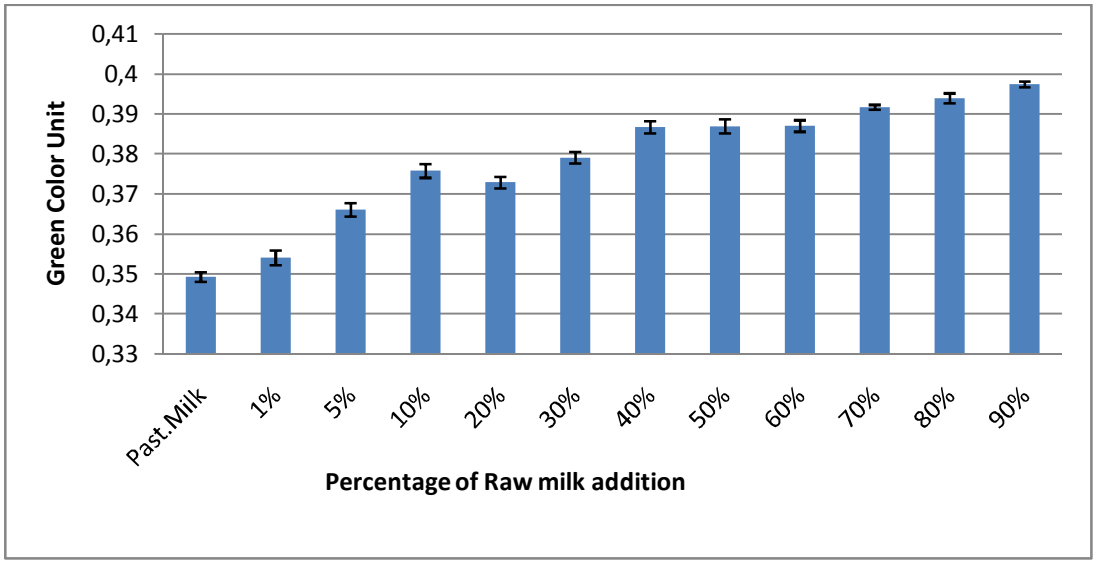

Figure3.58. The analysis of green color intensity ratio of bioreporters for the detection limit of mixing of pasteurized milk with raw milk from Kırıkkale. Vertical bars represent standart error of mean (SEM) and mean values with SEM values are tabulated at Table B. 31 in Appendix B.

3.7 The Study of Different Support Materials

Throughout the construction studies of bioreporter, the Whatman filter papers were used as support material and substrate-chromogen mixture was absorbed onto these papers. In order to determine the most effective support material the cotton of ear cleaning rod and bandage were also tested as support material. The color formations on these support material after the application of milk samples were evaluated as the responses of these bioreporters. These responses have been shown in Figure 3. 59.

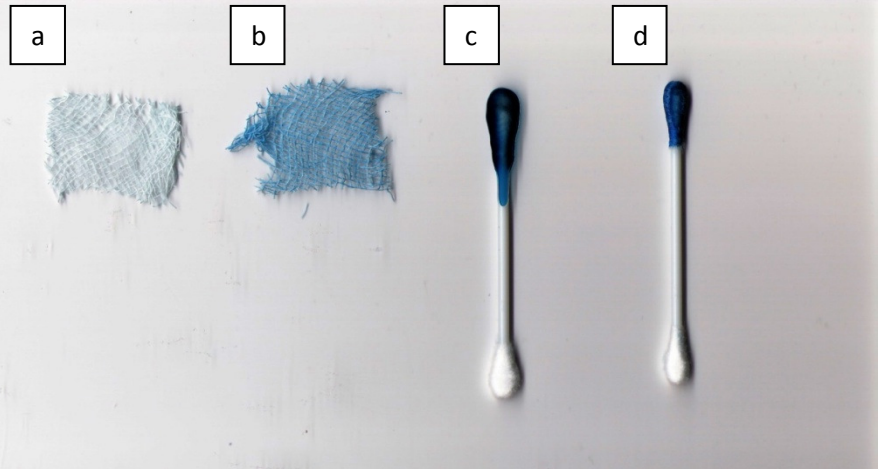

Figure 3.59. The effects of different support materials to the response of bioreporters. a. bandage applied with raw milk, b. bangade applied with UHT milk, c. cotton applied with raw milk, d. cotton applied with UHT milk.

Although the bioreporter constructed with the cotton of ear cleaning rod and bandage showed green color formation upon the application of milk containing 0.5 U/mL of alkaline phosphatase, these support materials are not applicable in bioreporter production. In the consequence of having more porous structure, the cotton was absorbed more substrate-chromogen mixture to saturate these pores. The increase in the volume of substrate and chromogen mixture leads to raise in the cost of bioreporter production. Another support material ,the bandage, has higher pore size and this makes the absorption of substrate and chromogen mixture difficult. According to these drawbacks of cotton and bandage, the Whatman filter paper was determined as an effective and applicable support material for the construction of alkaline phosphatase bioreporter.

3.8 The Study of Shelf Life Conditions of Bioreporter

In this study, the stability of bioreporter constructed with the optimum subsrate and chromogen concentration was tested at two different temperatures (4˚C and room temperature). At 10 days intervals bioreporters were tested with UHT milk and milk sample containing 0.5 U/mL of alkaline phosphatase. The change in the color on papers from blue to green upon the application of milk samples was evaluated as the response of bioreporter. Also, the change in the main color of paper during storage time was evaluated without any application of milk samples.

Figure 3.60 shows the stability of constructed papers at 4˚C and room temperature for 70 days. After 10 days at room temperature and 40 days storage time at 4˚C the color of paper was turning to green gradually without the action of alkaline phosphatase in milk. p-NPP absorbed on papers was decomposing into yellow product (p-NP) nonenzymatically. The yellow colored product mixed with blue colored chromogen and as a result, green color formation was observed on papers. The already existing green color on papers prevented the observation of green color resulting from the alkaline phosphatase activity in milk. The response of bioreporter to UHT milk was also observed as green color after the storage time of 10 days at room temperature and 40 days at 4˚C.

Based on this result, the optimum condition for the stability and thus the shelf life of bioreporter was determined as 4˚C for 40 days.

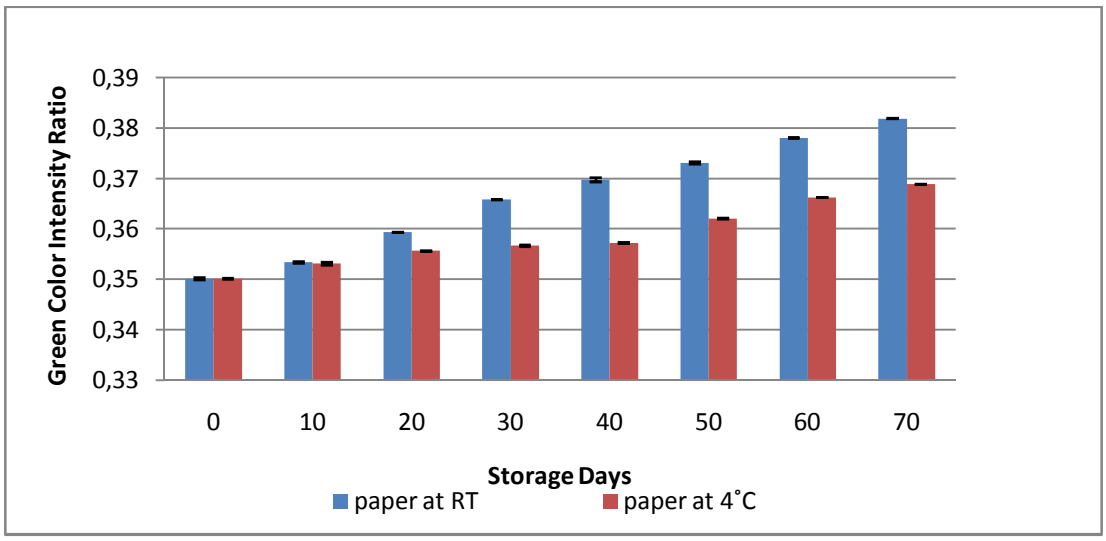

Figure 3.60. The analysis of green color intensity ratio of bioreporters stored at 4˚C and room temperature for 70 days. Vertical bars represent standart error of mean (SEM) and mean values with SEM values are tabulated at Table B. 32 in Appendix B.

3.9 Enzymatic Assay of Alkaline phosphatase

In this study, the enzyme assay of alkaline phosphatase in milk was performed in order to correlate the unit of alkaline phosphatase in milk found by assay with the response of bioreporter. Milk samples containing standard concentration of alkaline phosphatase (0.1 -10.0 U/mL of ALP) were assayed with 1 mg/mL of p-NPP solution in 0.1 M glycine buffer with 1.0 mM magnesium chloride and 1.0 mM zinc chloride at pH 9.5. The specrophotometric results were monitored for 5 min at 405 nm and used to plot a standard graph of alkaline phosphatase assay in milk (Figure 3.61).

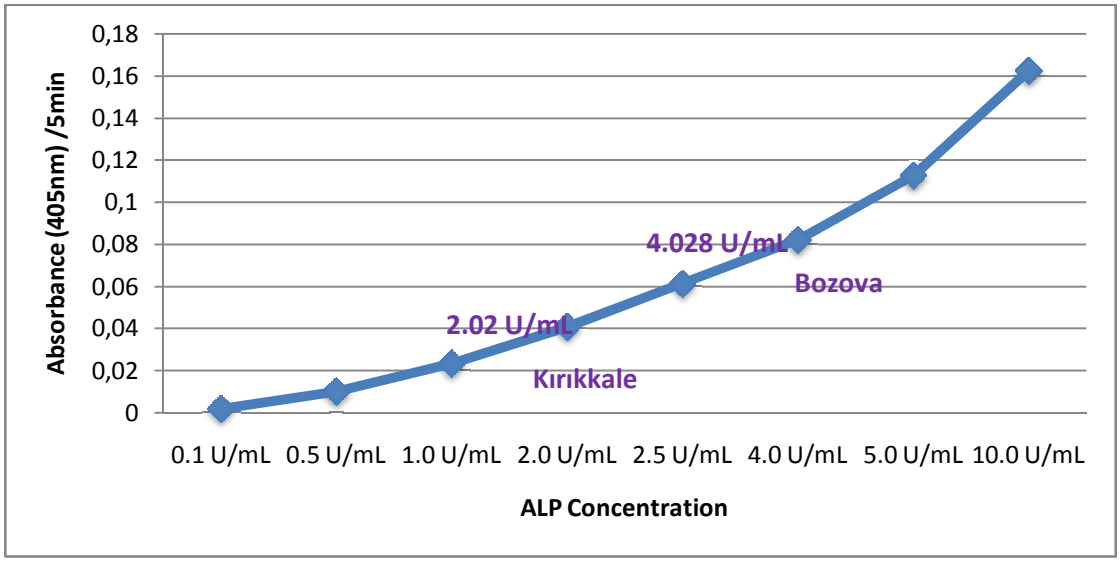

Figure 3.61. The standart curve of alkaline phosphatase assay in milk. Vertical bars represent standart error of mean (SEM) and mean values with SEM values are tabulated at Table B. 33 in Appendix B.

After the construction of standard curve of alkaline phosphatase, raw milk samples from Kırıkkale and Bozova were measured spectrophotometrically based on their alkaline phosphatase activity. According to their absorbance values per 5 min, the alkaline phosphatase activity in these two raw milk samples were determined by the standard formula. The ALP activities of raw milk from Kırıkkale and Bozova are shown in Figure 3.61.

CHAPTER 4

CONCLUSION

In this study, the development of a bioreporter for the process control in pasteurization of milk was targeted. The semiquantitative determination of alkaline phosphatase which is the reporter for the unproper pasteurization process in milk was performed with the paper based alkaline phosphatase bioreporter. Providing advantages over standard techniques such as being easy to use, cheap and practical, alkaline phosphatase bioreporter was targeted to fullfill the requirements of markets.

For the construction of alkaline phosphatase bioreporter, the optimization studies of substrate and chromogen were performed and as a result, 1 mg/mL of p-NPP in 0.1 M glycine buffer at pH 9.5 and 0.5 mg/mL of bromocresol green in 1 M Tris-HCl buffer at pH 9.5 were determined as optimum concentrations.

The alkaline phosphatase activity in milk at 0.5 U/mL and higher values was detected by bioreproter as a clear green color formed on papers which were constructed with optimum concentrations of p-NPP and bromocresol green. This detection limit included the standard stated by Turkish Standard Institution. Although the bioreporter does not give any quantitative results based on the enzyme concentration in milk, it can be used to evaluate the pasteurization process according to color change without any qualified staff and instrumentation.

After the determination of detection limit, the effect of sample temperature and pH values were tested on the bioreporter constructed according to the optimum substrate and chromogen concentration. The response of bioreporter which is the color change on paper upon sample application was not affected from the temperature of milk. The milk samples which were stored at 4˚C, room temperature and 37˚C leaded to the formation of green color on papers with at least 0.5 U/mL of alkaline phosphatase activity in milk. In addition to the temperature, the effect of sample pH on the response of bioreporter was also tested. The milk samples at pH 5, 5.5, 6 and 6.5 were applied to constructed bioreporter and the color changes showed that our bioreporter can be used to evaluate pasteurization process of milk at the pH value between 5 to 6.5.

Moreover, the effectiveness of bioreporter was tested for milk samples from different animals including cattle, sheep and goat and from different locations in Turkey. The raw milk samples from sheep and cattle resulted in the green color formation on papers while raw goat milk sample did not result in a change in color since the alkaline phosphatase activity was lower in goat milk compared with sheep and cattle. As expected, our bioreporter gave appropriate response only to unpasteurized sheep and cattle milk. In addition, raw milk samples from different locations in Turkey were tested for their alkaline phospatase activity and the response of bioreporter ensured the applicability of bioreporter to different milk samples.

The heat treatment of standart and raw milk samples were performed at 50˚C and 60˚C for 30 min and the residual alkaline phosphatase activity in these samples were determined with this paper based bioreporter according to the green color formation on papers. The effectiveness of pasteurization process was evaluated with the responses of bioreporter. The application of heat at 50 ˚C for 30 min

leaded to the decrease in the activity of alkaline phosphatase in samples, however, the end products of treatment were still unpasteurized milk. The evaluation of pasteurization was provided by appropriate response of our bioreporter. After the treatment of milk samples at 60˚C for 30 min, the decrease in alkaline phosphatase activity was higher compared with treatment at 50˚C. This results were also provided with bioreporter as the decrease in green color intensity ratio.

The effectiveness of bioreporter was also tested for the detection limit of raw milk mixed in pasteurized end product. The commertial UHT milk was mixed with raw milk samples at a range between 1 to 90 % and the green color formations on constructed papers were evaluated. According to the green color intensity ratio analysis, the addition of 1% raw milk to pasteurized milk was detected by bioreporter. However, based on visual inspection, our bioreporter can be used to detect at least 10% mixing of raw milk to pasteurized milk.

In the study, different support materials were used to determine the most applicable support material. In addition to Whatman filter papers, cotton and bandage were absorbed with optimum substrate and chromogen mixture and milk samples were applied on them. The increase in the volume of mixture for the saturation of cotton resulted in higher production cost. The higher pore size of bandage brought on difficulty in the absorption of mixture on bandage. In the consequences of these reasons, Whatman filter paper was an effective and applicable support material for the construction of alkaline phosphatase bioreporter.

Finally, the stability test was performed at 4˚C and room temperature in order to determine the shelf life of bioreporter. While the bioreporter at 4˚C room temperature did not give appropriate response after 40 days, it lost its stability at

room temperature after 10 days of storage time. At room temperature after 10 days p-NPP was decomposed into its yellow product spontaneously without the action of alkaline phosphatase in milk and the color of paper was changing from blue to green gradually during the storage time at room temperature. Therefore, the response of bioreporter stored at room temperature to milk samples containing alkaline phosphatase was not appropriate. The decomposition of p-NPP was observed also in papers stored at 4˚C after the storage time of 40 days. Since the optimum stability of papers was observed at 4˚C, the shelf life condition for the alkaline phosphatase bioreporter was determined as 40 days at 4˚C.

In future studies, in order to increase the stability and thus the shelf life of bioreporter, certain stabilizers should be immobilized to papers with substrate and chromogen. The solid support materials and certain adhesives should be tested for the construction of alkaline phosphatase bioreporter as a marketing study. Finally, the quantification studies of alkaline phosphatase in milk should be performed to support the applicability of bioreporter.

REFERENCES

Abad J. M., Pariente F., Hernandez L., Abrun˜a H. D., Lorenzo E., 1998, Determination of organophosphorus and carbamate pesticides using a piezoelectric biosensor, Analytical Chemistry, 70, 14, 2848–2855

Ahmed M. U., Hossain M. M., Tamiyaa E., 2008, Electrochemical Biosensors for Medical and Food Applications, Electroanalysis, 20, 6, 616 – 626

Ama´rita, F., Ferna´ndez, C. R., Alkorta, F., 1997, Hybrid biosensors to estimate lactose in milk, Analytica Chimica Acta, 349, 1–3, 153–158

Amine A., Mohammadi H., Palleschi G., 2006, Enzyme inhibition-based biosensors for food safety and environmental monitoring, Biosensors and Bioelectronics, 21, 1405–1423

Andreou V.G., Clonis Y.D., 2002, Novel fiber-optic biosensor based on immobilized glutathione S-transferase and sol–gel entrapped bromocresol green for the determination of atrazine, Anal. Chim. Acta, 460, 151–161

Andrews A. T., Anderson M., Goodnough P. W., 1987, A study of the heat stabilities of number of indigenous milk enzymes, Journal of Dairy Research, 54, 237–246

Arakawa H., Shiokawa M., Imamura O., Maeda M., 2003, Novel bioluminescent assay of alkaline phosphatase using adenosine-3′-phosphate-5′-phosphosulfate as substrate and the luciferin–luciferase reaction and its application, Anal. Biochem, 314, 206-211

Babacan S., Pivarnik P., Letcher S., Rand A. G., 2000, Evaluation of antibody immobilization methods of piezoelectric biosensor application, Biosensors and Bioelectronics, 15, 11–12, 615–621

Balcer H.I, Kwon H.J, Kang K.A, 2002, Assay procedure optimization of a rapid, reusable protein C immunosensor for physiological samples, Ann Biomed Eng., 30, 141–147

Bassi A. S., Lee E., Zhu J.X, 1998, Carbon paste mediated amperometric thin film biosensors for fructose monitoring in honey, Food Research International, 31, 2, 119–127

Becker T, Hitzmann B, Muffler K, Pörtner R, Reardon KF, Stahl F, Ulber R., 2007, Future aspects of bioprocess monitoring. Adv Biochem Eng Biotechnol.,105, 249-93

Bizet K., Gabrielli C., Perrot H., 1999, Biosensors based on piezoelectric transducers, Analusis, 27, 7, 609–616

Boujtita M., Murr N.El., 2000, Ferrocene-mediated carbon paste electrode modified with D-fructose dehydrogenase for batch mode measurement of D-fructose, Applied BioChemistry and BioTechnology, 89, 1, 55–66

Boujtita M., Hart J. P., Pittson R., 2000, Development of a disposable ethanol biosensor based on a chemically modified screen-printed electrode coated with alcohol oxidase for the analysis of beer. Biosensors and Bioelectronics, 15, 5–6, 257–263

Bunde R. L., Jarvi E. J., Rosentreter J. J., 1998, Piezoelectric quartz crystal biosensors, Talanta, 46, 1223-1236

Carter R. M., Jacobs M. B., Lubrano G. J., Guilbault, G. G., 1997, Rapid detection of aflatoxin B1 with immunochemical optodes, Analytical Letters, 30, 8, 1465–1482

ChaÂvarri F., Santisteban A., Virto M., de Renobales M., 1998, Alkaline Phosphatase, Acid Phosphatase, Lactoperoxidase, and Lipoprotein Lipase Activities in Industrial Ewe's Milk and Cheese, J. Agric. Food Chem., 46, 2926−2932

Chaplin M.F. (2000). Biosensors. In J.M.Walker, R.Rapley (Eds.), Molecular Biotechology and Biotechnology (pp: 521-555). Cambridge: Royal Society of Chemistry.

Chen C. C., Tai Y. C., Shen S. C., Tu Y. Y., Wu M. C., Chang H. M., 2006, Detection of alkaline phosphatase by competitive indirect ELISA using immunoglobulin in yolk (IgY) specific against bovine milk alkaline phosphatase, Food Chemistry, 95, 213–220

Chouteau C., Dzyadevych S., Chovelon J. M., Durrieu C., 2004, Development of novel conducto-metric biosensors based on immobilised whole cell chlorella vulgaris microalgae. Biosensors &Bioelectronics, 19, 9, 1089–1096

Ciafardini G., Zullo B.A., 1998, Assay of microbial enzymes in opaque samples, Journal of Microbiological Methods, 34, 73–79

Claeys W. L., Ludikhuyze L. R., Loey A. M. V., Hendrickx M. E., 2001, Inactivation kinetics of alkaline phosphatase and lactoperoxidase, and denaturation kinetics of b-lactoglobulin in raw milk under isothermal and dynamic temperature conditions, Journal of Dairy Research, 68, 95-107

Claeys W. L., Loey A. M. V., Hendrickx M. E., 2003, Influence of seasonal variation on kinetics of time temperature integrators for thermally processed milk, Journal of Dairy Research, 70, 217–225

Claeys W. L., Indrwati, Loey A. M. V., Hendrickx M. E., 2003, Review: are intrinsic TTIs for thermally processed milk applicable for high-pressure processing assessment?, Innovative Food Science and Emerging Technologies, 4, 1–14

Clark L. Jr, Lyons C., 1962, Electrode systems for continuous monitoring in cardiovascular surgery. Ann N Y Acad Sci, 102, 29– 45

Curulli A., Kelly S., O'Sullivan C. O., Guilbault G. G., Palleschi G., 1998, A new interference-free lysine biosensor using a non-conducting polymer film, Biosensors and Bioelectronics, 13, 12, 1245–1250

Decristoforo, G., Danielsson, B., 1984, Flow injection analysis with enzyme thermistor detector for automated determination of α-lactams. Anal. Chem., 56, 263–268

Delory G. E., Royal Infirmary, Preston, King E. J., 1945, A Sodium Carbonate-bicarbonate Buffer for Alkaline Phosphatases, British Postgraduate Medical School, London, 39, 245

Dinnella C., Monteleone E., Farenga M. F., Hourigan J. A., 2004, The use of enzymes for thermal process monitoring: modification of milk alkaline phosphatase heat resistance by means of an immobilization technique, Food Control, 15, 427–433

D'Orazio P., 2003, Biosensors in clinical chemistry, Clinica Chimica Acta, 334, 41–69

Fadıloğlu S., Erkmen O., Şekerolu G., 2006, THERMAL INACTIVATION KINETICS OF ALKALINE PHOSPHATASE IN BUFFER AND MILK, Journal of Food Processing and Preservation, 30, 258–268

Fenoll J., Jourquin G., Kauffmann J. M., 2002, Fluorimetric determination of alkaline phosphatase in solid and fluid dairy products, Talanta, 56, 1021–1026

Ferreira L.S., De Souza M.B. Jr, Trierweiler J.O., Broxtermann O., Folly R.O.M., Hitzmann B., 2003, Aspects concerning the use of biosensors for process control: experimental and simulation investigations, Computers and Chemical Engineering, 27, 1165-1173

Fox P.F., Kelly A.L., 2006, Indigenous enzymes in milk: Overview and historical aspects—Part 2, International Dairy Journal, 16, 517–532

Frense D., Müller A. and Beckmann D., 1998, Detection of environmental pollutants using optical biosensor with immobilized algae cells , Sensors and Actuators B: Chemical, 51(1-3) , 256-260

Gajovic N., Warsinke A., Scheller F. W., 1997, Comparison of two enzymes sequences for a novel l-malate biosensor, Journal of Chemistry Technology and BioTechnology, 68, 31–36

Garcia C. A. B., Neto G. O., Kubota L. T., 1998, New fructose biosensors utilizing a polypyrrole film and D-fructose 5-dehydrogenase immobilized by different processes, Analytica Chimica Acta, 374, 2–3, 201–208

Gauglitz G., 2005, Direct optical sensors: principles and selected applications, Anal Bioanal Chem, 381, 141–155

Gehring A.G., Brewster J.D., Irwin P.L., Tu S., Van Houten L.J., 1999, 1-Naphthyl phosphate as an enzymatic substrate for enzyme-linked immunomagnetic electrochemistry, *J. Electroanal. Chem,* 469**,** 27–33

Geneix N., Dufour E., Venien A., Levieux D., 2007, Development of a monoclonal antibody-based immunoassay for specific quantification of bovine milk alkaline phosphatase, Journal of Dairy Research, 74, 290–295

Grieshaber D., MacKenzie R., Vörös J., Reimhult E., 2008, Electrochemical Biosensors -Sensor Principles and Architectures, Sensors, 8, 1400-1458

Griffiths M. W., 1986, Use the milk enzymes as indices of heat treatment, Journal of Food Protection, 49, 696–705

Gu M.B., Gil G.C., Kim J.H., 1999, A two-stage minibioreactor system for continuous toxicity monitoring, Biosens Bioelectron, 14, 355–361

Gu M.B., Gil G.C., 2001, A multi-channel continuous toxicity monitoring system using recombinant bioluminescent bacteria for classification of toxicity, Biosens Bioelectron, 16, 661–666

Guilbault G., 1984, Handbook of Immobilized Enzymes, Marcel Dekker, New York, 87

Hall R. H., 2002, Biosensor technologies for detecting microbiological foodborne hazards, Microbes and Infection, 4, 425–432

Hamid I. A., Ivnitski D., Atanasov P., Wilkins E., 1999, Highly sensitive flow-injection immunoassay system for rapid detection of bacteria, Analytica Chimica Acta, 399, 1–2, 99–108

Hava N., Ariel L., 1980, The Activation of Alkaline Phosphatase by Effector Molecules. A Combined Kinetic and Hydrodynamic Study, European journal of biochemistry, 109, 2 , 475-480

Hitzmann B., Broxtermann O., Cha Y.-L., Sobieh O., Sta¨rk E., Scheper T., 2000, The control of glucose concentration during yeast fed-batch cultivation, Bioprocess Engineering , 23, 4, 337-341

Horacek J., Garrett S. D., Skla´dal P., Morgan M. R. A., 1998, Characterization of the interactions between immobilized parathion and the corresponding recombinant scFv antibody using a piezoelectric biosensor, Food and Agricultural and Immunology, 10, 4, 363–374

Ishikawa E., 1987, Development and clinical application of sensitive enzyme immunoassay for macromolecular abtigens- a review, Clin. Biochem, 20, 375-385

Ivnitski D., Hamid I. A., Atanasov P., Wilkins E., 1999, Biosensors for detection of pathogenic bacteria, Biosensors and Bioelectronics, 14, 7, 599–624

Katrlı´k J., Svorc J., Stred'ansky´ M., Miertus S., 1998, Composite alcohol biosensors based on solid binding matrix, Biosensors and Bioelectronics, 13, 2, 183–191

Kelly A.L., Fox P.F., 2006, Indigenous enzymes in milk: A synopsis of future research requirements, International Dairy Journal, 16, 707–715

Kokado A., Tsuji A., Maeda M., 1997, Chemiluminescence assay of alkaline phosphatase using cortisol-21 -phosphate as substrate and its application to enzyme immunoassays, Anal. Chim. Acta, 337, 335-340

Kreuzer M.P., O'Sullivan C.K., Guilbault G.G., 1999, Alkaline phosphatase as a label for immunoassay using amperometric detection with a variety of substrates and an optimal buffer system, Anal. Chim. Acta, 393, 95-102

Kueng A., Kranz C., Mizaikoff B., 2004, Amperometric ATP biosensor based on polymer entrapped enzymes, Biosensors & Bioelectronics, 19, 10, 1301–1307

Levieux D., Geneix N., Levieux A., 2007, Inactivation-denaturation kinetics of bovine milk alkaline phosphatase during mild heating as determined by using a monoclonal antibody-based immunoassay, Journal of Dairy Research, 74, 296–301

Lianidou E.S. , Ioannou P.C. , Sacharidou E. , 1994, Second derivative synchronous scanning fluorescence spectrometry as a sensitive detection technique in immunoassays, Application to the determination of α-fetoprotein, Anal. Chim. Acta, 290, 159-165

Lin J.M., Tsuji A., Maeda M., 1997, Chemiluminescent flow injection determination of alkaline phosphatase and its applications to enzyme immunoassays, Anal. Chim. Acta, 339, 139-146

Ljutovac R. K., Park Y. W., Gaucheron F., Bouhallab S., 2007, Heat stability and enzymatic modifications of goat and sheep milk, Small Ruminant Research, 68, 207–220

Ludikhuyze L., Claeys W., Hendrickx M., 2000, Combined Pressure–temperature Inactivation of Alkaline Phosphatase in Bovine Milk: A Kinetic Study, JOURNAL OF FOOD SCIENCE, 65, 1, 155-160

Luong J. H. T., Bouvrette P., Male K. B., 1997, Developments and applications of biosensors in food analysis,TIBTECH, 15, 369-377

Luong J. H. T., Mulchandani A., Guilbault G. G., 1988, Developments and applications of biosensors, Trends in BioTechnology, 6, 12, 310–316

Mandenius C., Bu¨low L., Danielsson B., Mosbach K., 1985, Monitoring and control of enzymic sucrose hydrolysis using online biosensors, Appl. Microbiol. Biotechnol., 21, 135–141

Mao L., Yamamoto K., 2000, Amperometric on-line sensor for continuous measurement of hypoxanthine based on osmium-polyvinylpyridine gel polymer and xanthine oxidase bienzyme modified glassy carbon electrode, Analytica Chimica Acta, 415, 1–2, 143–150

Marazuela M. D., Moreno-Bondi M. C., 2002, Fiber-optic biosensors – an overview, Anal Bioanal Chem, 372 , 664–682

Masson M., Haruyama T., Kobatake E., Aizawa M., 1999, 4-Hydroxynaphthyl-1-phosphate as a substrate for alkaline phosphatase and its use in sandwich immunoassay Anal. Chim. Acta, 402, 29

Masson M.,. Runarsson O.V, Johannson F., Aizawa M., 2004, 4-Amino-1-naphthylphosphate as a substrate for the amperometric detection of alkaline phosphatase activity and its application for immunoassay, Talanta, 64, 174–180

Matsumuto K., Asada W., Murai R., 1998, Simultaneous biosensing of inosine monophosphate and glutamate by use of immobilized enzyme reactors, Analytica Chimica Acta, 858, 2, 127–136

Mattiasson B., Danielsson B., 1982, Calorimetric analysis of sugars and sugar derivatives with aid of an enzyme thermistor, Carbohydr. Res., 102, 273–282

Mattiasson B., Danielsson B., Hermannsson C., Mosbach K., 1978, Enzyme thermistor analysis of heavy metal ions with use of immobilized urease, FEBS Lett., 85, 203–206

McKellar A. T., Emmons D. B., 1991, g-Glutamyl transpeptidase in milk and butter as indicator of heat treatment, International Dairy Journal, 1, 241–251

Medyantseva E. P., Vertlib M. G., Budnikov G. K., Tyshlek M. P., 1998, A new approach to selective detection of 2,4-diclorophenoxyacetic acid with a cholinesterase amperometric biosensor, Applied Biochemistry and Microbiology, 34, 2, 202–205

Mehrab M., Bıs C., Scharer J. M., Young M. M., Luong J. H., 2000, Fiber-Optic Biosensors- Trends and Advances, Analytical Science, 16, 677-692

Mello L. D., Kubota L. T., 2002, Analytical, Nutritional and Clinical Methods,Review of the use of biosensors as analytical tools in the food and drink industries, Food Chemistry, 77, 237–256

Monk D. J., Walt D. R., 2004, Optical fiber-based biosensors, Anal Bioanal Chem, 379, 931–945

Mortier L., Braekman A., Cartuyvels D., Renterghem R. V., Block J.D., 2000, Intrinsic indicators for monitoring heat damage of consumption milk, Biotechnol. Agron. Soc. Environ., 4, 4, 221–225

Mussa D. M., Ramaswamy H. S., 1997, Ultra High Pressure Pasteurization of Milk: Kinetics of Microbial Destruction and Changes in Physico-chemical Characteristics, Lebensm.-Wiss. u.-Technol., 30, 551–557

Narayanaswamy R., 2006, Optical chemical sensors and biosensors for food safety and security applications, Acta Biologica Szegediensis, 50,3-4, 105-108

Newman J. D., Tigwell L. J., Turner A. P. F., Warner P., 2004, J.Biosensors: A clearer view. 8th World Congress on Biosensors, Granada, Spain, 24-26 May

Palchetti I., Cagnini A., Del Carlo M., Coppi C., Mascini M., Turner A. P. F., 1997, Determination of anticholinesterase pesticides in real samples using a disposable biosensor, Analytica Chimica Acta, 337, 3, 315–321

Picart L., Thiebaud M., Rene M., Guiraud J. P., Cheftel J. C., Dumay E., 2006, Effects of high pressure homogenisation of raw bovine milk on alkaline phosphatase and microbial inactivation. A comparison with continuous short-time thermal treatments, Journal of Dairy Research, 73, 454–463

Pita M. T. P., Reviejo A. J., Villena F. J. M., Pingarro´n J. M, 1997, Amperometric selective biosensing of dimethyl-and diethyl-dithiocarbamates based on inhibition processes in a medium of reversed micelles, Analytica Chimica Acta, 340, 1–3, 89–97

Preininger C., Danielsson B., 1996, Thermometric determination of copper (II) using acid urease, Analyst, 121, 1717–1720

Privett B. J., Shin J.H., Schoenfisch M. H., 2008, Electrochemical Sensors, Anal. Chem. , 80, 4499–4517

Prodromidis M. I., Karayannis M. I., 2002, Enzyme Based Amperometric Biosensors for Food Analysis, Electroanalysis, 14, 4, 241-261

Ramanathan K., Rank M., Svitel J., Dzgoev A., Danielsson B., 1999, The development and applications of thermal biosensors for bioprocess monitoring, TIBTECH, 17, 499-505

Ramanathan K., Svitel J., Dzgoev A., Sundaram P.V., Danielsson B., 2000, Biomaterials for molecular electronics: development of an optical biosensor for retinol., Appl. Biochem. Biotechnol.,96,1-3,287-301

Ramanathan K., Danielsson B., 2001, Principles and applications of thermal biosensors, Biosensors & Bioelectronics, 16, 417–423

Rampling A. M., Greenwood M. H., Davies G. E. N., 2004, Use of a fluorimetric test for bovine alkaline phosphatase to demonstrate under-pasteurisation of skimmed milk and cream, International Dairy Journal, 14, 691–695

Rasooly A., Rasooly L., 1999, Real time biosensor analysis of Staphylococcal enterotoxin A in food, International Journal of Food Microbiology, 49, 3, 119–127

Riverol C., Ricart G., Carosi C., Di Santis C., 2008, Application of advanced soft control strategies into the dairy industry, Innovative Food Science and Emerging Technologies, 9, 298– 305

Serraa B. , Moralesb M.D., Reviejob A.J., Halla E.H., Pingarróna J.M., 2005, Rapid and highly sensitive electrochemical determination of alkaline phosphatase using a composite tyrosinase biosensor, Analytical Biochemistry, 336 ,289–294

Scheller F. W., Wollenberger U., Warsinke A., Lisdat F., 2001, Research and development in biosensors, Analytical biotechnology, 12, 35–40

Shamsi K., Versteeg C., Sherkat F., Wan J., 2008, Alkaline phosphatase and microbial inactivation by pulsed electric field in bovine milk, Innovative Food Science and Emerging Technologies, 9, 217– 223

Sharma S. K., Sehgal N., Kumar A., 2003, Biomolecules for development of biosensors and their applications, Current Applied Physics, 3, 307-316

Sharma S. K., Sehgal N., Kumar A., 2003, Dry-reagent strips for testing milk pasteurization, Lebensm.-Wiss. u.-Technol., 36, 567–571

Sherlock M., Fu B., Taoukis P.S., Labuza T.P., 1991, A systematic evaluation of time–temperature indicators for use as consumer tags, Journal of Food Protection 54, 11, 885–889

Stinson R.A., Chan J.R.A., 1987, Alkaline phosphatase and its function as a protein phosphatase, Adv. Protein Phosphatases, 4, 127-151

Stradiotto N. R., Yamanaka H. , Zanoni M. V. B., 2003, Electrochemical Sensors: A Powerful Tool in Analytical Chemistry, J. Braz. Chem. Soc., 14, 2, 159-173.

Su X., Low S., Kwang J., Chew V. H. T., Li S. F. Y., 2001, Piezoelectric quartz crystal based veterinary diagnosis for Salmonella enteritidis infection in chicken and egg, Sensors and Actuators B, 75, 1–2, 29–35

Terefe N.S., Arimi J.M., Van Loey A., Hendrickx M., 2004, Kinetics of the alkaline phosphatase catalyzed hydrolysis of disodium p-nitrophenyl phosphate: effects of carbohydrate additives, low temperature, and freezing, Biotechnol. Prog., 20, 1467-1478

Terry L.A., White S. F., Tigwell L. J., 2005, The Application of Biosensors to Fresh Produce and the Wider Food Industry, J. Agric. Food Chem., 53, 1309−1316

Thévenot D.R., Toth K., Durst R.A., Wilson G.S., 2001, Electrochemical biosensors:recomended definitions and classification, Biosensors & Bioelectronics, 16, 121-131

Turkish Standard, TS 1019, 2002, Turkish Standard Institution

Vamvakaki A. N., Zoidou E., Moatsou G. Bokari M. Anifantakis E., 2006, Residual alkaline phosphatase activity after heat treatment of ovine and caprine milk, Small Ruminant Research, 65, 237–241

Van Loey A., Hendrickx M., De Cordt S., Haentjens P., Tobback P., 1996, Quantitative evaluation of thermal processes using time–temperature integrators, Trends in Food Science & Technology, 7, 16–25

Vega-Warner A. V., Gandhi H., Smith D. M., Ustunol Z., 2000, Polyclonal-Antibody-Based ELISA To Detect Milk Alkaline Phosphatase, J. Agric. Food Chem., 48, 2087−2091

Wang J., 1999, Amperometric biosensors for clinical and therapeutic drug monitoring: a review, Journal of Pharmaceutical and Biomedical Analysis, 19, 47-53

Wilin´ska A., Bryjak J, Illeova´ V., Polakovicˇ M., 2007, Kinetics of thermal inactivation of alkaline phosphatase in bovine and caprine milk and buffer, International Dairy Journal, 17, 579–586

Wilkinson J.H., Vodden A.V., Phenolphthalein Monophosphate as a Substrate for Serum Alkaline Phosphatase, Clin. Chem., 12, 1966, 701-708

Winquist F., Danielsson B., Malpote J-Y., Larsson M-B., 1985, Determination of oxalate with immobilized oxalate oxidase in an enzyme thermistor, Anal. Lett., 18, 573–588

Wu T-Z., 1999, A piezoelectric biosensor as an olfactory receptor for odour detection: electronic nose., Biosensors and Bioelectronics, 14, 1, 9–18

Xie B., Harborn U., Mecklenburg M., Danielsson B., 1994, Urea and lactate determined in 1-µl whole blood samples with a miniaturized thermal biosensor, Clin. Chem, 40, 2282–2287

Yagiuda K., Hemmi A., Ito S., Asano Y., Fushinuki Y., Chen C. Y., Karube I., 1996, Development of a conductivity-based immunosensor for sensitive detection of methamphetamine (stimulant drug)in human urine. Biosensors & Bioelectronics, 11, 8, 703–707

Yoshitomi K., 2004, Alkaline phosphatase activity in cheeses measured by fluorometry, International Journal of Food Science and Technology, 39, 349–353

Zhu X., Liu Q., Jiang C., 2006, 2-Carboxy-1-naphthyl phosphate as a substrate for the fluorimetric determination of alkaline phosphatase, Analytica Chimica Acta, 570, 29–33

Zhu X., Liu Q., Jiang C., 2007, 8-Quinolyl phosphate as a substrate for the fluorimetric determination of alkaline phosphatase, Clinica Chimica Acta, 377, 150– 153

APPENDIX A

REACTION MECHANISM OF ALKALINE PHOSPHATASE

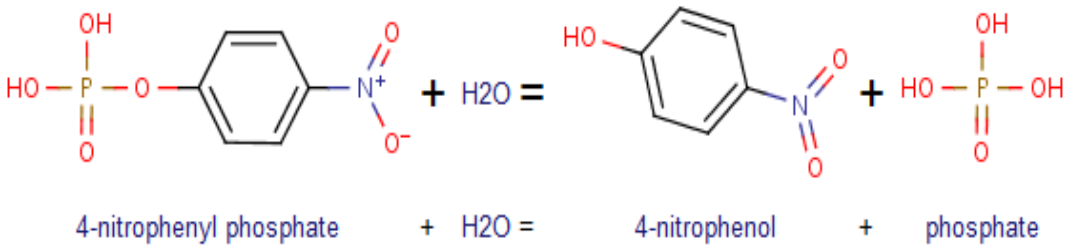

Figure A.1. Reaction mechanism of alkaline phosphatase with its substrate, p-NPP (Terefe *et al*., 2004).

Basic Principle of Bİoreporter

In alkaline pH, the phosphate group of p-nitrophenly phosphate is cleaved by the action of alkaline phosphatase. The end product , p-nitrophenol, has yellow color. The bromocresol green which is a dye of the triphenylmethane family ionize in aqueous solution to give blue color at alkaline pH and yellow color at acidic pH. At alkaline pH provided with glycine buffer at pH 9.5 blue colored bromocresol green and yellow colored p-nitrophenol form green color.

APPENDIX B

TABULATED VALUES OF GREEN COLOR INTENSITY RATIO

Table B.1. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M sodium carbonate buffer at pH 9.0. (Figure 3.2) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34553 ±0,000165	0,34838± 0,00008	0,34600 ± 0,000018
0.5U/mL	0,35701 ±0,000471	0,35369± 0,000051	0,34576 ± 0,000169
1.0U/mL	0,37468 ±0,000223	0,37206± 0,000075	0,34689 ± 0,000033
5.0U/mL	0,37039 ±0,000299	0,37223± 0,000149	0,34963 ± 0,000374
10.0U/mL	0,38058 ±0,000226	0,34894± 0,000457	0,34836 ± 0,000301
Pasteurized milk	0,34274 ±0,000056	0,34402± 0,000033	0,34214 ± 0,000112

Table B.2. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M sodium carbonate buffer at pH 9.5. (Figure 3.4) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34330 ±0,000012	0,34616 ±0,000271	0,35057 ±0,000109
0.5U/mL	0,36134 ±0,000081	0,36002 ±0,000106	0,34758 ±0,000124
1.0U/mL	0,37354 ±0,000169	0,35103 ±0,000403	0,34841 ±0,000021
5.0U/mL	0,38660 ±0,000609	0,36741 ±0,000233	0,35602 ±0,000156
10.0U/mL	0,37618 ± 0,00136	0,34146 ±0,000232	0,34765 ±0,000278
Pasteurized milk	0,33957 ±0,000071	0,34279 ±0,000290	0,34209 ±0,000134

Table B.3. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M sodium carbonate buffer at pH 10.0. (Figure 3.6) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,35466 ± 0,000087	0,35080 ± 0,000216	0,34805 ± 0,000144
0.5U/mL	0,38925 ± 0,000063	0,36678 ± 0,000142	0,34777 ± 0,000034
1.0U/mL	0,39279 ± 0,000295	0,37944 ± 0,000106	0,35208 ± 0,000391
5.0U/mL	0,40659 ± 0,000162	0,38008 ± 0,000497	0,35516 ± 0,000209
10.0U/mL	0,39839 ± 0,000077	0,37289 ± 0,000127	0,35353 ± 0,000049
Pasteurized milk	0,34143 ± 0,000041	0,34319 ± 0,000020	0,34455 ± 0,000149

Table B.4. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M sodium carbonate buffer at pH 10.5. (Figure 3.8) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34695 ± 0,000108	0,34178 ± 0,000035	0,33687 ±0,0000142
0.5U/mL	0,3534 ± 0,000049	0,34391 ± 0,000056	0,33957 ± 0,000132
1.0U/mL	0,34475 ± 0,000121	0,35129 ± 0,000102	0,34271 ± 0,000076
5.0U/mL	0,35535 ± 0,000179	0,34237 ± 0,000156	0,34562 ± 0,000134
10.0U/mL	0,35221 ± 0,000126	0,35109 ± 0,000103	0,34128 ± 0,000021
Pasteurized milk	0,34154 ± 0,000252	0,34101 ± 0,000201	0,33367 ± 0,000064

Table B.5. Alkaline phosphatase concentration versus p-NPP concentration prepared in 1.0 M diethanolamine buffer at pH 9.0. (Figure 3.10) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34567 ± 0,000038	0,35110 ± 0,000144	0,34602 ± 0,000041
0.5U/mL	0,34878 ± 0,000358	0,35825 ± 0,000265	0,34990 ± 0,000020
1.0U/mL	0,35191 ± 0,000671	0,36344 ± 0,000107	0,34827 ± 0,000222
5.0U/mL	0,36142 ± 0,000039	0,36852 ± 0,000045	0,34682 ± 0,000098
10.0U/mL	0,35517 ± 0,000075	0,36140 ± 0,000023	0,35000 ± 0,000034
Pasteurized milk	0,34166 ± 0,000045	0,34201 ± 0,000031	0,34388 ± 0,000076

Table B.6. Alkaline phosphatase concentration versus p-NPP concentration prepared in 1.0 M diethanolamine buffer at pH 9.5. (Figure 3.12) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,35375 ± 0,000048	0,34875 ± 0,000068	0,34696 ± 0,000018
0.5U/mL	0,38108 ± 0,000313	0,37716 ± 0,000127	0,35346 ± 0,000043
1.0U/mL	0,37336 ± 0,000209	0,36649 ± 0,000380	0,35476 ± 0,000076
5.0U/mL	0,37519 ± 0,000144	0,36160 ± 0,000139	0,35341 ± 0,000125
10.0U/mL	0,37967 ± 0,000121	0,36043 ± 0,000254	0,34878 ± 0,000122
Pasteurized milk	0,34363 ± 0,000133	0,34537 ± 0,000020	0,34464 ± 0,000052

Table B.7. Alkaline phosphatase concentration versus p-NPP concentration prepared in 1.0 M diethanolamine buffer at pH 10.0. (Figure 3.14) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34775 ± 0,000026	0,34951 ± 0,000079	0,34457 ± 0,000024
0.5U/mL	0,36661 ± 0,000160	0,35468 ± 0,000024	0,34721 ± 0,000024
1.0U/mL	0,36784 ± 0,000233	0,35325 ± 0,000362	0,34785 ± 0,000040
5.0U/mL	0,37336 ± 0,000282	0,35819 ± 0,000001	0,34764 ± 0,000114
10.0U/mL	0,37329 ± 0,000031	0,36122 ± 0,000105	0,34555 ± 0,000047
Pasteurized milk	0,34283 ± 0,000047	0,34229 ± 0,000111	0,34130 ± 0,000050

Table B.8. Alkaline phosphatase concentration versus p-NPP concentration prepared in 1.0 M diethanolamine buffer at pH 10.5. (Figure 3.16) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,35663 ± 0,000064	0,35398 ± 0,000168	0,34605 ± 0,000060
0.5U/mL	0,38648 ± 0,000042	0,36426 ± 0,000267	0,34575 ± 0,000204
1.0U/mL	0,38118 ± 0,000210	0,36996 ± 0,000010	0,34937 ± 0,000140
5.0U/mL	0,37861 ± 0,000157	0,36428 ± 0,000321	0,35077 ± 0,000296
10.0U/mL	0,38359 ± 0,000156	0,36647 ± 0,000137	0,34516 ± 0,000044
Pasteurized milk	0,34369 ± 0,000081	0,34314 ± 0,000048	0,34201 ± 0,000039

Table B.9. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M glycine buffer at pH 9.0. (Figure 3.18) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34553 ± 0,000165	0,34599 ± 0,000115	0,34356 ± 0,000152
0.5U/mL	0,35701 ± 0,000471	0,35107 ± 0,000103	0,34726 ± 0,000091
1.0U/mL	0,37468 ± 0,000223	0,35140 ± 0,000171	0,34458 ± 0,000216
5.0U/mL	0,37039 ± 0,000299	0,34907 ± 0,000171	0,34628 ± 0,000250
10.0U/mL	0,38058 ± 0,000226	0,34948 ± 0,000040	0,34425 ± 0,000094
Pasteurized milk	0,34274 ± 0,000056	0,34457 ± 0,000107	0,34424 ± 0,000106

Table B.10. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M glycine buffer at pH 9.5. (Figure 3.20) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34701 ± 0,000066	0,34598 ± 0,000160	0,34558 ± 0,000160
0.5U/mL	0,35916 ± 0,000118	0,35261 ± 0,000039	0,34784 ± 0,000102
1.0U/mL	0,36655 ± 0,000137	0,35754 ± 0,000005	0,35143 ± 0,000021
5.0U/mL	0,37150 ± 0,000356	0,35965 ± 0,000310	0,34586 ± 0,000025
10.0U/mL	0,38856 ± 0,000622	0,36598 ± 0,000045	0,34412 ± 0,000027
Pasteurized milk	0,34443 ± 0,000070	0,34358 ± 0,000177	0,34384 ± 0,000133

Table B.11. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M glycine buffer at pH 10.0. (Figure 3.22) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,35007 ± 0,000030	0,34373 ± 0,000024	0,34251 ± 0,000042
0.5U/mL	0,35900 ± 0,000160	0,35387 ± 0,000079	0,34652 ± 0,000193
1.0U/mL	0,36729 ± 0,000152	0,34844 ± 0,000158	0,34437 ± 0,000060
5.0U/mL	0,36731 ± 0,000358	0,34775 ± 0,000143	0,34651 ± 0,000197
10.0U/mL	0,37018 ± 0,000148	0,34714 ± 0,000198	0,34802 ± 0,000282
Pasteurized milk	0,34667 ± 0,000047	0,34329 ± 0,000138	0,34375 ± 0,000126

Table B.12. Alkaline phosphatase concentration versus p-NPP concentration prepared in 0.1 M glycine buffer at pH 10.5. (Figure 3.24) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio		
	Substrate Concentration		
	2mg/mL	1mg/mL	0.5mg/mL
0.1U/mL	0,34987 ± 0,00002	0,34428 ± 0,000172	0,34121 ± 0,000111
0.5U/mL	0,35899 ± 0,000033	0,35137 ± 0,000106	0,34571 ± 0,000124
1.0U/mL	0,36252 ± 0,000069	0,34538 ± 0,000043	0,34372 ± 0,000031
5.0U/mL	0,36331 ± 0,000209	0,34459 ± 0,000135	0,34524 ± 0,000196
10.0U/mL	0,36763 ± 0,000153	0,34413 ± 0,000232	0,3472 ± 0,000278
Pasteurized milk	0,34317 ± 0,000094	0,34139 ± 0,00029	0,34125 ± 0,000146

Table B.13. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 0.1 M glycine buffer at pH 8.0. (Figure 3.26) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,36708 ± 0,000131	0,37159 ± 0,000269	0,37196 ± 0,000086	0,34753 ± 0,000040
0.5U/mL	0,35970 ± 0,000100	0,37860 ± 0,000107	0,38181 ± 0,000163	0,35198 ± 0,000210
1.0U/mL	0,36667 ± 0,000227	0,38010 ± 0,000064	0,38070 ± 0,000116	0,35483 ± 0,000256
5.0U/mL	0,36828 ± 0,000011	0,38640 ± 0,000218	0,39092 ± 0,000028	0,35494 ± 0,000168
10.0U/mL	0,36576 ± 0,000384	0,39282 ± 0,000099	0,39287 ± 0,000266	0,36043 ± 0,000240
Pasteurized milk	0,34829 ± 0,000120	0,37774 ± 0,000521	0,37975 ± 0,000010	0,34749 ± 0,000128

Table B.14. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 0.1 M glycine buffer at pH 8.5. (Figure 3.28) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,36249 ± 0,000131	0,37098 ± 0,000269	0,37132 ± 0,000086	0,34665 ± 0,000040
0.5U/mL	0,35679 ± 0,000100	0,37688 ± 0,000107	0,38109 ± 0,000163	0,35091 ± 0,000210
1.0U/mL	0,36221 ± 0,000227	0,38103 ± 0,000064	0,38057 ± 0,000116	0,35386 ± 0,000256
5.0U/mL	0,36828 ± 0,000011	0,38640 ± 0,000218	0,39092 ± 0,000028	0,35494 ± 0,000168
10.0U/mL	0,36576 ± 0,000384	0,39282 ± 0,000099	0,39287 ± 0,000266	0,36043 ± 0,000240
Pasteurized milk	0,34829 ± 0,000120	0,37774 ± 0,000521	0,37975 ± 0,000010	0,34749 ± 0,000128

Table B.15. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 0.1 M glycine buffer at pH 9.0. (Figure 3.30) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,35926 ± 0,000062	0,36846 ± 0,000147	0,37194 ± 0,000075	0,34257 ± 0,000118
0.5U/mL	0,35911 ± 0,000089	0,37569 ± 0,000131	0,37107 ± 0,000010	0,35007 ± 0,000045
1.0U/mL	0,36128 ± 0,000113	0,38064 ± 0,000065	0,37190 ± 0,000098	0,34706 ± 0,000590
5.0U/mL	0,36681 ± 0,000064	0,38713 ± 0,000065	0,37724 ± 0,000099	0,34733 ± 0,000031
10.0U/mL	0,36482 ± 0,000204	0,38559 ± 0,000028	0,38570 ± 0,000111	0,35202 ± 0,000174
Pasteurized milk	0,36176 ± 0,000202	0,36749 ± 0,000100	0,37024 ± 0,000297	0,34255 ± 0,000156

Table B.16. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 0.1 M glycine buffer at pH 9.5. (Figure 3.32) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,37518 ± 0,000145	0,37702 ± 0,000055	0,37528 ± 0,000061	0,34738 ± 0,000131
0.5U/mL	0,38039 ± 0,000043	0,38336 ± 0,000119	0,37983 ± 0,000032	0,35741 ± 0,000183
1.0U/mL	0,38509 ± 0,000068	0,38392 ± 0,000042	0,38340 ± 0,000269	0,35836 ± 0,000081
5.0U/mL	0,38551 ± 0,000248	0,39307 ± 0,000065	0,38050 ± 0,000076	0,36276 ± 0,000952
10.0U/mL	0,37837 ± 0,000117	0,38351 ± 0,000121	0,35953 ± 0,000159	0,35831 ± 0,000576
Pasteurized milk	0,37534 ± 0,000523	0,37833 ± 0,000232	0,36823 ± 0,000026	0,34975 ± 0,000426

Table B.17. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 1.0 M Tris-Hcl buffer at pH 8.0. (Figure 3.34) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,34641 ± 0,000117	0,37382 ± 0,000105	0,39181 ± 0,000051	0,35096 ± 0,000113
0.5U/mL	0,35746 ± 0,000039	0,38694 ± 0,000075	0,40633 ± 0,000053	0,35493 ± 0,000186
1.0U/mL	0,35815 ± 0,000038	0,39442 ± 0,000035	0,40828 ± 0,000031	0,35869 ± 0,000566
5.0U/mL	0,36063 ± 0,000036	0,39762 ± 0,000117	0,39161 ± 0,000029	0,35226 ± 0,000066
10.0U/mL	0,36762 ± 0,000123	0,40121 ± 0,000060	0,39660 ± 0,000037	0,36379 ± 0,000045
Pasteurized milk	0,34197 ± 0,000455	0,36662 ± 0,000028	0,38598 ± 0,000009	0,34506 ± 0,000093

Table B.18. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 1.0 M Tris-Hcl buffer at pH 8.5. (Figure 3.36) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,35840 ± 0,000149	0,38810 ±0,000072	0,39550 ± 0,000030	0,35038 ±0,000089
0.5U/mL	0,36886 ± 0,000109	0,40460 ± 0,000041	0,40998 ± 0,000032	0,35936 ± 0,000471
1.0U/mL	0,37838 ± 0,000051	0,41165 ± 0,000298	0,41394 ± 0,00211	0,37330 ± 0,000380
5.0U/mL	0,37725 ± 0,000025	0,41131 ± 0,000216	0,41293 ± 0,000198	0,38067 ± 0,000198
10.0U/mL	0,38250 ± 0,000035	0,40583 ± 0,000030	0,41609 ± 0,000064	0,37803 ± 0,000700
Pasteurized milk	0,33075 ± 0,000007	0,36791 ± 0,000497	0,38575 ± 0,000042	0,34745 ± 0,000177

Table B.19. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 1.0 M Tris-Hcl buffer at pH 9.0. (Figure 3.38) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,34310 ± 0,000545	0,38109 ± 0,000051	0,39401 ± 0,000345	0,34934 ± 0,000154
0.5U/mL	0,36365 ± 0,000025	0,40365 ± 0,000069	0,41111 ± 0,000048	0,36048 ± 0,000617
1.0U/mL	0,37541 ± 0,000078	0,40860 ± 0,000066	0,41961 ± 0,000228	0,37446 ± 0,000121
5.0U/mL	0,37343 ± 0,000046	0,40837 ± 0,000074	0,40765 ± 0,000008	0,37369 ± 0,000150
10.0U/mL	0,37778 ± 0,000094	0,40916 ± 0,000022	0,40826 ± 0,000008	0,36506 ± 0,000952
Pasteurized milk	0,32751 ± 0,000127	0,38044 ± 0,000363	0,38561 ± 0,000420	0,34434 ± 0,000254

Table B.20. Alkaline phosphatase concentration versus bromocresol green concentration prepared in 1.0 M Tris-Hcl buffer at pH 9.5. (Figure 3.40) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in milk	Green Color Intensity Ratio			
	Chromogen Concentration			
	2mg/mL	1mg/mL	0.5mg/mL	0.1mg/mL
0.1U/mL	0,34438 ± 0,000078	0,38398 ± 0,000088	0,38952 ± 0,000158	0,34879 ± 0,000073
0.5U/mL	0,36023 ± 0,000039	0,39792 ± 0,000247	0,40842 ± 0,000299	0,37101 ± 0,000127
1.0U/mL	0,37377 ± 0,000061	0,40452 ± 0,000012	0,39825 ± 0,000289	0,37950 ± 0,000200
5.0U/mL	0,37789 ± 0,000014	0,40923 ± 0,000162	0,41255 ± 0,000048	0,37624 ± 0,000086
10.0U/mL	0,38475 ± 0,000091	0,41128 ± 0,000186	0,40750 ± 0,000117	0,37282 ± 0,000433
Pasteurized milk	0,33396 ± 0,000387	0,37614 ± 0,000572	0,38880 ± 0,000107	0,34425 ± 0,000042

Table B.21. Alkaline phosphatase concentration for the determination of detection limit (Figure 3.42). Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in Milk	Green Color Intensity Ratio
0.005 U/mL	0,36455 ± 0,000134
0.01 U/mL	0,37728 ± 0,000132
0.05 U/mL	0,38952 ± 0,000158
0.1 U/mL	0,40842 ± 0,000299
0.5 U/mL	0,39825 ± 0,000289
1.0 U/mL	0,41255 ± 0,000048
5.0 U/mL	0,40750 ± 0,000117
10.0 U/mL	0,38880 ± 0,000107
Control (glycine buffer)	0,33220 ± 0,000007
UHT	0,35527 ± 0,000073

Table B.22. Alkaline phosphatase concentration versus sample temperature. (Figure 3.44) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in Milk	Green Color Intensity Ratio		
	Sample temperature		
	37˚C	4˚C	RT
0.1U/mL	0,37486 ± 0,000205	0,39553 ±0,000090	0,37845 ±0,000013
0.5U/mL	0,39967 ± 0,000116	0,38476 ± 0,000139	0,39477 ± 0,000056
1.0U/mL	0,39792 ± 0,000068	0,38421 ± 0,000072	0,38839 ± 0,000072
5.0U/mL	0,40979 ± 0,000410	0,37375 ± 0,000186	0,39788 ± 0,000051
Control (glycine buffer)	0,35249 ± 0,000077	0,34895 ± 0,000054	0,35182 ± 0,000047
UHT	0,38174 ± 0,000137	0,37185 ± 0,000117	0,37500 ± 0,000056

Table B.23. Alkaline phosphatase concentration versus sample pH. (Figure 3.46) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in Milk	Green Color Intensity Ratio			
	Sample pH			
	pH 5.0	pH 5.5	pH 6.0	pH 6.5
0.1U/mL	0,36935 ± 0,000067	0,37193 ± 0,000291	0,37546 ± 0,000177	0,38174 ± 0,000034
0.5U/mL	0,38429 ± 0,000123	0,38576 ± 0,000148	0,38776 ± 0,000153	0,39792 ± 0,000121
1.0U/mL	0,38875 ± 0,000049	0,38846 ± 0,000037	0,38881 ± 0,000043	0,39967 ± 0,000017
5.0U/mL	0,39979 ± 0,000071	0,40187 ± 0,000021	0,40465 ± 0,000055	0,40979 ± 0,000175
Control (glycine buffer)	0,35122 ± 0,000034	0,35131 ± 0,000082	0,35162 ± 0,000156	0,35149 ± 0,000129
UHT	0,37491 ± 0,000176	0,37479 ± 0,000014	0,37481 ± 0,000022	0,37486 ± 0,000037

Table B.24. Milk samples from different animal sources. (Figure 3.48) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Raw milk samples	Green Color Intensity Ratio
Cattle milk	0,39880 ± 0,000268
Goat milk	0,38527 ± 0,000019
Sheep milk	0,41026 ± 0,000135
Control (glycine buffer)	0,35249 ± 0,000021
UHT	0,37482 ± 0,000128

Table B.25. Lipid free milk samples from different animal source. (Figure 3.50) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Raw milk samples	Green Color Intensity Ratio
Cattle milk	0,38597 ± 0,000044
Lipid free Cattle milk	0,38468 ± 0,000013
Goat milk	0,36875 ± 0,000107
Lipid free goat mik	0,36362 ± 0,000115
Sheep milk	0,40216 ± 0,000031
Lipid free sheep milk	0,40312 ± 0,000103
Control (glycine buffer)	0,34019 ± 0,000026
UHT	0,35782 ± 0,000132
Lipid free UHT	0,35799 ± 0,000211

Table B.26. Milk samples from different location in Turkey. (Figure 3.52) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Raw milk samples from	Green Color Intensity Ratio
Kırıkkale	0,38972 ± 0,000105
Yahyalı	0,38449 ± 0,000037
Varollar	0,38608 ± 0,000066
Çamlık	0,37569 ± 0,000123
Bozova	0,37965 ± 0,000147
Kutludüğün	0,38741 ± 0,000041
Konya	0,37409 ± 0,000018
Burdur	0,37353 ± 0,000033
Control (glycine buffer)	0,34528 ± 0,000025
UHT	0,3614 ± 0,000111

Table B.27. Alkaline phosphatase concentration versus treatment time of milk at 50˚C (Figure 3.53) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in Milk	Green Color Intensity Ratio			
	0 min	10 min	20 min	30 min
0.1U/mL	0,37584± 0,000108	0,36807 ± 0,000023	0,368 ± 0,000171	0,36232 ± 0,000076
0.5U/mL	0,39202 ± 0,000032	0,38588 ± 0,000063	0,37937 ± 0,000301	0,37172 ± 0,000132
1.0U/mL	0,39492 ± 0,000117	0,39253 ± 0,00014	0,38618 ± 0,000044	0,38755 ± 0,000132
5.U/mL	0,3979 ± 0,000145	0,39393± 0,000012	0,39237 ± 0,000208	0,38658 ± 0,000093
10.0U/mL	0,39924 ± 0,000061	0,39435 ± 0,000152	0,38547 ± 0,000071	0,38241 ± 0,000103
Past. Milk	0,3658 ± 0,000024	0,36588 ± 0,000173	0,36381 ± 0,000039	0,36299 ± 0,0000121

Table B.28. Alkaline phosphatase concentration versus treatment time of milk at 60˚C (Figure 3.54) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Enzyme Concentration in Milk	Green Color Intensity Ratio			
	0 min	10 min	20 min	30 min
0.1U/mL	0,37484 ± 0,000118	0,36888 ± 0,000052	0,3684 ± 0,000127	0,36185 ± 0,000096
0.5U/mL	0,39122 ± 0,000093	0,37292 ± 0,000046	0,3696 ± 0,000209	0,36564 ± 0,000132
1.0U/mL	0,39475 ± 0,00012	0,37837 ± 0,000174	0,37391 ± 0,000056	0,36487 ± 0,000111
5.U/mL	0,39679 ± 0,00019	0,3788 ± 0,000182	0,37767 ± 0,000203	0,3714 ± 0,000079
10.0U/mL	0,39847 ± 0,000026	0,38265 ± 0,000135	0,37429 ± 0,000021	0,37095 ± 0,000153
Past. Milk	0,36151 ± 0,000052	0,36122 ± 0,000187	0,36076 ± 0,000064	0,35966 ± 0,000021

Table B.29. Milk samples from different location in Turkey versus treatment time of milk at 50˚C (Figure 3.55) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Raw Milk Samples	Green Color Intensity Ratio			
	0 min	10 min	20 min	30 min
Kırıkkale	0,38388 ± 0,000422	0,38145 ± 0,000112	0,37219 ± 0,000133	0,36954 ± 0,000119
Yahyalı	0,38015 ± 0,000186	0,37785 ± 0,000105	0,37762 ± 0,000046	0,37345 ± 0,000738
Varollar	0,38322 ± 0.000096	0,38092 ± 0,000183	0,37828 ± 0,000361	0,37442 ± 0,000278
Çamlık	0,38243 ± 0,000396	0,37594 ± 0,000256	0,37411 ± 0,000165	0,37035 ± 0,00048
Bozova	0,38329 ± 0,000777	0,38311 ± 0,000192	0,38304 ± 0,000346	0,3763 ± 0,000398
Kutludüğün	0,37699 ± 0,000204	0,37521 ± 0,000107	0,37509 ± 0,000157	0,37317 ± 0,000245
UHT	0,36125 ± 0,000075	0,3595 ± 0,00008	0,35742 ± 0,000608	0,35691 ± 0,000918

Table B.30. Milk samples from different location in Turkey versus treatment time of milk at 60˚C (Figure 3.56) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Raw Milk Samples	Green Color Intensity Ratio			
	0min	10min	20min	30min
Kırıkkale	0,38644± 0,000242	0,37096 ± 0,000105	0,3662 ± 0,000063	0,36256 ± 0,000019
Yahyalı	0,37421 ± 0,000096	0,37186 ± 0,000109	0,37328 ± 0,000226	0,36952 ± 0,000231
Varollar	0,38608± 0,000068	0,38051 ± 0,000183	0,37415 ± 0,000165	0,36444 ± 0,000048
Çamlık	0,37659 ± 0,000396	0,37547 ± 0,000256	0,37367 ± 0,000165	0,36339 ± 0,000048
Bozova	0,37965 ± 0,000077	0,37567 ± 0,000172	0,37216 ± 0,000157	0,36452 ± 0,000245
Kutludüğün 0,000154	0,38534 ± 0,000154	0,37944 ± 0,000107	0,37371 ± 0,000157	0,36184 ± 0,000245
UHT	0,36014 ± 0,000135	0,35876 ± 0,000118	0,3555 ± 0,000068	0,35434 ± 0,000189

Table B.31. The detection limit of the addition of raw milk to UHT milk .(Figure 3.58) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Addition of Raw Milk to UHT	Green Color Intensity
UHT	0,34927 ± 0,001229
1%	0,35408 ± 0,001792
5%	0,36606 ± 0,00169
10%	0,37575 ± 0,0017
20%	0,37289 ± 0,001452
30%	0,37906 ± 0,001446
40%	0,38673 ± 0,001538
50%	0,38691 ± 0,00176
60%	0,387 ± 0,001436
70%	0,39171 ± 0,000565
80%	0,39391 ± 0,00124
90%	0,39742 ± 0,000689

Table B.32. The stability of bioreporter at 4˚C and room temperature .(Figure 3.60) Mean values are obtained from tripcate readings and the reported errors are standart error of mean (SEM).

Stability of Constructed Paper	Green Color Intensity	
Storage Days	4˚C	Room Temperature
0	0,35019 ± 0,000143	0,35021 ± 0,000224
10	0,35317 ± 0,000256	0,35344 ± 0,000192
20	0,35569 ± 0,000024	0,35938 ± 0,000069
30	0,35672 ± 0,000189	0,36587 ± 0,000071
40	0,35728 ± 0,000091	0,36985 ± 0,000431
50	0,36214 ± 0,000112	0,37319 ± 0,000246
60	0,36631 ± 0,000042	0,37811 ± 0,000138
70	0,36893 ± 0,000058	0,38193 ± 0,000035

Table B.33. The measurement of standard alkaline phosphatase activity based on absorbance at 405 nm per 5 min versus alkaline phosphatase activity.(Figure 3.61) Mean values are obtained from tripcate readings.

ALP concentration	Absorbance at 405 nm / 5 min
0.1 U/mL	0,00206
0.5 U/mL	0,0103
1.0 U/mL	0,0236
2.0 U/mL	0,041
2.5 U/mL	0,0614
4.0 U/mL	0,0822
5.0 U/mL	0,1128
10.0 U/mL	0,1625

Table B.34. The measurement of alkaline phosphatase activity in raw milk samples from Kırıkkale and Bozova based on absorbance at 405 nm per 5 min versus alkaline phosphatase activity.(Figure 3.61) Mean values are obtained from tripcate readings.

Raw milk samples	Absorbance at 405 nm / 5 min
Kırıkkale	0,08281
Bozova	0,04159