EFFECT OF pH CHANGES ON THE GROWTH OF SELECTED BACTERIA IN COCONUT MILK

by

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CERTIFICATE

This is to certify that the dissertation entitled "EFFECT OF pH CHANGES ON THE GROWTH OF SELECTED BACTERIA IN COCONUT MILK" is the bonafide record of research work done by Ms Marahaini bt Musa during the period from July 2009 to October 2009 under my supervision.

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List of symbol and abbreviation

C	Control
TSI	Triple Sugar Iron
MR/VP	Methyl Red/ Voges-Proskauer
HCL	Hydrochloric acid
NaOH	Sodium hydroxide

Abstrak

Kelapa (*Cocos nucifera*) dijumpai tumbuh di kawasan tanah rendah, tropika dan subtropika, telah digunakan sebagai sumber berguna bukan sahaja untuk makanan tetapi juga untuk tujuan perubatan. Salah satu produk daripada kelapa diguna sebagai ubat adalah santan kelapa. Santan kelapa adalah ekstrak akueous daripada endosperma kelapa. Ia merupakan makanan rendah asid, dengan pH sekitar 6.2 dan kaya dengan nutrien. Sifat-sifat santan kelapa ini menyediakan medium yang sesuai untuk pertumbuhan mikroorganisma. Cara sterilisasi yang berkesan diaplikasi untuk menghapuskan mikroorganisma yang merbahaya, menjadikan santan kelapa selamat untuk dimakan. Amnya, salah satu teknik untuk mengawet makanan adalah menggunakan bahan kimia. Bahan ini termasuklah asid and bes yang mengubah pH makanan and menyekat pertumbuhan sesetengah bakteria. Dalam kajian ini, kami mengkaji kesan perubahan pH terhadap bakteria terpilih yang hidup dalam santan kelapa. Sampel santan kelapa telah dibahagikan kepada kumpulan berlainan pH, dari asid kepada alkali, bermula dari pH 10, 8, 6, 4 dan 2. Sampel kontrol disediakan tanpa dimasukkan sebarang bahan kimia. Sampel diinokulasi ke atas media MacConkey dan MRS untuk megenal pasti bakteria yang hidup dalam santan kelapa. Morphologi bakteria dikaji dengan mikroskop dan ujian biokimia dijalankan untuk identifikasi bakteria. Penurunan pH direkodkan sepanjang eksperimen kecuali bagi kumpulan pH 2. Kami mendapati perubahan pH telah memberi kesan kepada kadar pertumbuhan bakteria dalam santan kelapa. *Klebsiella pnemoniae*, *Acenitobacter* dan *Staphylococcus* lebih sesuai hidup pada keadaan alkali di mana pertumbuhan *Lactobacillus* lebih baik pada pH berasid. Maklumat yang dikumpul dapat memberi petunjuk yang lebih jelas ke arah aktiviti yang memerlukan pengetahuan tentang pertumbuhan bakteria, contohnya perumusan semula medium untuk pertumbuhan bakterium terpilih.

Abstract

Coconuts (*Cocos nucifera*) are found growing around the world in lowland, tropical and subtropical habitats and have been used as a valuable source not only for food but also as medicine. One of the coconut's product used as medicine is coconut milk. Coconut milk is the aqueous extract of the solid coconut endosperm. It is a low-acid liquid food, with a pH of around 6.2 and very rich in nutrient. These properties of coconut milk provides suitable medium for growth of microorganisms. Effective methods of sterilization are practiced to eliminate harmful microorganism, making coconut milk safe to be consumed. A preservation technique used in general food preservation is by the addition of chemicals. These chemicals include acids and bases which alter pH of food and inhibit growth of certain bacteria. In this research, we study effect of pH changes on selective bacteria growing in coconut milk. Coconut milk samples are subjected to different pH ranging from acidic to alkaline, started from pH 10, 8, 6, 4 and 2. Control sample is prepared without addition of chemicals. Sample is inoculated on MacConkey and MRS agar in order to identify selective bacteria growing in coconut milk. Morphology of bacteria is examined under microscope and biochemical tests are conducted for identification of bacteria. pH reductions are recorded throughout the experiment except for pH 2 group. Bacteria count (CFU/ml) is recorded over time. We found that pH changes affect the growth rate of bacteria in coconut. *Klebsiella pnemoniae*, *Acenitobacter* and *Staphylococcus* prefer alkaline condition whereas *Lactobacillus* grows better at acidic pH. Gathered information could provide clearer guidance towards activities which need information about bacterial growth, for example in development of new media for growth of selected bacteria.

Chapter 1: Introduction

1.1 Coconut milk

Coconuts (*Cocos nucifera*) belong to the Palm family *Arecaceae* and are found growing around the world in lowland, tropical and subtropical habitats (Ray, 2002). According to the website of Coconut Research *Center*, nearly one third of the world's population depends on coconut to some degree for their food and their economy.

Coconut is highly nutritious and rich in fiber, vitamins, and minerals. It is classified as a "functional food" because it provides many health benefits beyond its nutritional content. The coconut palm is as highly valued as both a source of food and medicine that it is called "The Tree of Life."

People from many diverse cultures, languages, religions, and races scattered around the globe including South East Asian countries have used the coconut as a valuable source not only for food but also as medicine (Seow, 1997). The utilization of the different portions of the coconut tree for commercial matters makes it one of the most important crops in the tropics (Ray, 2002).

Around the world, coconuts have been used in traditional and modern medicine to treat wide variety of disease. One of the products of coconut which been practiced as medicine is coconut milk. In Ayurvedic herbal treatment, coconut milk is used to treat mouth ulcer (Coconut Milk, Wikipedia). This has been proven effective as coconut milk and water via macroscopic observation had protective effects on the ulcerated gastric mucosa in rats ((Nneli and Woyike, 2008).

Coconut milk is derived from the flesh of the coconut. Coconut milk is an indispensable ingredient in the traditional South East Asian cuisines and also a main component in the local kitchen in other parts of the world, for example Africa (Seow, 1997).

There appears to be increasing demand for the coconut milk, for use in the home and in the food industry, even though oil recovery remains the major concern in the coconut industry. It has been estimated that 25% of the world's output of coconuts is consumed as coconut milk (Gwee, 1988).

Coconut milk is aqueous extract of the solid coconut endosperm, a white opaque protein-oil-water emulsion. This liquid is obtained by pressing grated solid coconut endosperm, with or without addition of water or liquid endosperm, referred to as coconut water. Coconut milk is an emulsion and not physically stable. It separates into two layers; cream and serum layer within 5 to 10 hours after production (Tangsuphoom, 2005).

1.2 Chemistry of coconut milk

The chemical composition of coconut milk show very wide variations depends on many factors like variety of coconut, geographical location, cultural practices, maturity of the nut, method of extraction, and the degree of dilution with added water or liquid endosperm (Cancel, 1997).

According to Seow in 1997, he says that coconut milk contains carbohydrates, mainly sugars and starch, and minerals, such as phosphorus, calcium and potassium. The main proteins in coconut milk are albumins and globulins. The protein content of the

undiluted milk ranges from 5-10% on dry basis, but when the milk is filtered only about 30% of the protein is dissolved in the aqueous phase.

Water content and fat content of coconut milk varied depend on the method of extraction either with cold water (27–30 °C) or hot water (88–93 °C), as reported by Cancel (1997).

1.3 Microorganisms and its process in food

Microorganisms present in the food that we eat (Adams, 2000). These microorganisms are introduced into the food from different sources. The primary function of microorganisms in nature is self-perpetuation (Jay, 2005). During this process, the heterotrophs and autotrophs carry out following general reaction where organic matter like carbohydrate, protein and lipids are been converted to energy and inorganic compound. These compounds include nitrate and sulphates.

Organic matters come from human daily food supply. The raw-material has its own micro-flora, but different steps in the chain from raw-material to finished food product also introduce microorganisms.

1.4 Scope of the research

Coconut milk is a very suitable medium to support growth of microorganism. Many methods of sterilization like high temperature sterilization, low temperature (freezing) radiation and is used to eliminate bacteria particularly in food industries. Other method is use of chemicals. Chemicals like acid and alkali are useful in producing pH changes in coconut milk. This method effectively sterilizes coconut milk as bacteria survive differently, depending on the environment. Different pH provides different condition for bacterial growth.

1.5 The purpose of this work

Coconut milk is widely use especially in Africa and South East Asia as one of the main ingredient in cooking. Malaysian cuisines particularly embrace coconut milk due to its delicious taste and as thickener in desert or main course.

Often, women especially tend to employ method to increase the shelf life of certain especially ones that contain coconut milk. One of the methods is by alteration of pH in the food itself. Agent that been usually used is acidic in nature. That includes tamarind juice and 'asam keping'. Acidification provides an environment where it is not suitable for growth or multiplication of certain microorganisms.

Even though the method in preserving food has been applied for generations, little is known with the interaction occur between microorganisms in coconut milk and the effect of pH changes in extending the shelf life of food.

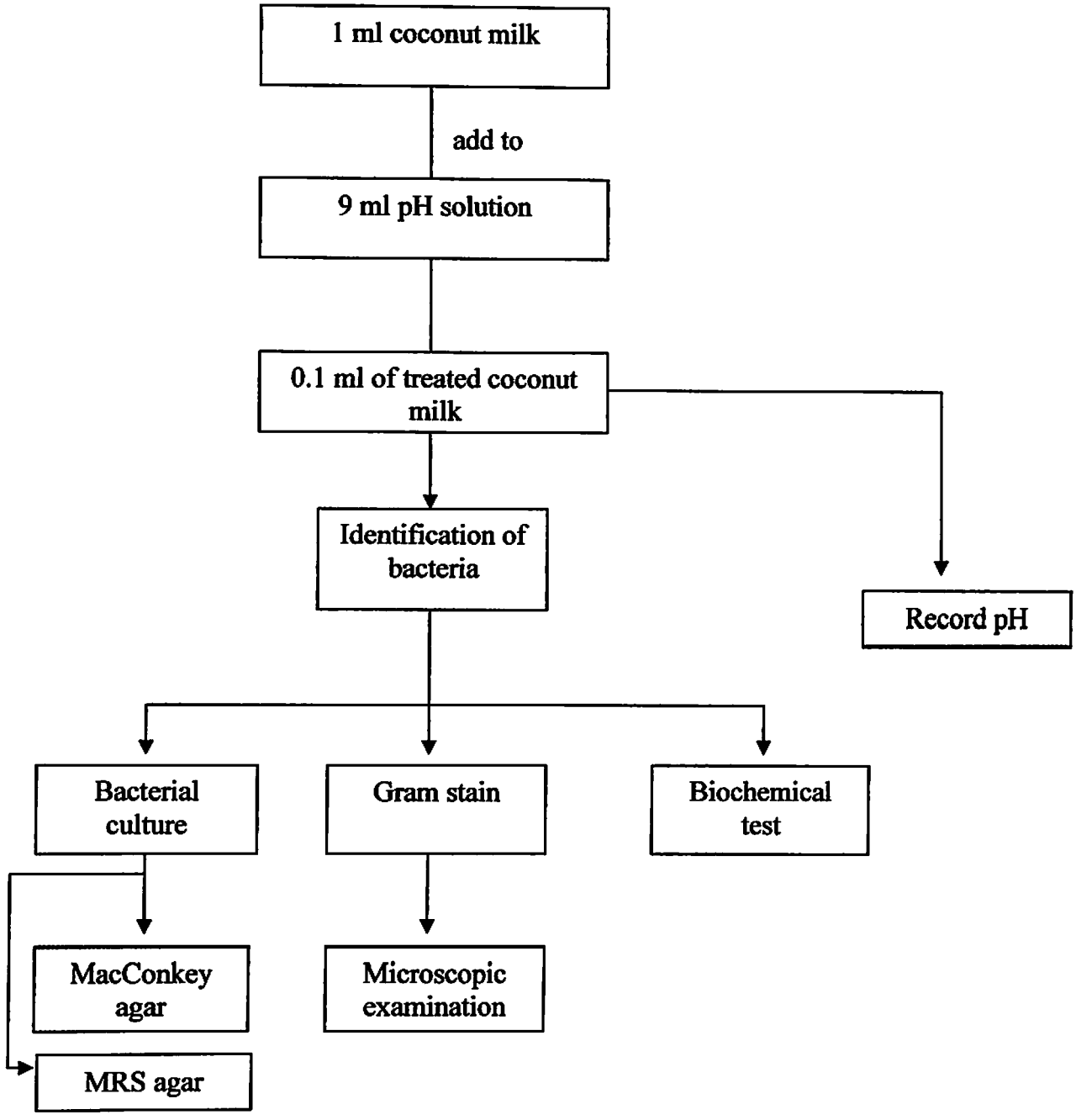
Therefore, the objectives of this study are:

1) To identify the bacteria that growing in exposed and environmentally non-exposed coconut milk.
2) To study the effect of pH changes on survival of selected bacteria growing in coconut milk.

1.6 Hypothesis

Growths of selected bacteria are affected by changing pH of coconut milk.

1.7 Flow chart of research experimental design



CHAPTER 2: LITERATURE REVIEW

2.1 Microorganisms growing in coconut milk

Coconut milk spoils rapidly even under chilled storage if it is not being treated (Seow, 1997). Coconut milk is a very rich medium which can support the growth of all the common spoilage microorganisms and spoils rapidly if unprocessed.

These microorganisms usually been introduced into coconut milk via contaminated shells, utensils, processing equipment and handlers (Seow, 1997). Shell of coconuts contain large population of microorganisms like *Acinetobacter, Enterobacteriaceae, Flavobacterium, Micro-bacterium, Micrococcus* species, yeasts and molds which potentially lead to coconut milk spoilage (Kajs *et al.*, 1975).

Three types of microorganisms are found to grow in coconut milk. They consist of bacteria, yeast and mould. Among bacteria that grow in coconut milk are *Bacillus, Micrococcus, Alcaligenes, Achromobacter, Streptococcus, Staphylococcus* and *Lactobacillus* (Jay, 2005), (Mepba, 2003), (Seow, 1997).

Some of the microorganisms isolated in coconut milk are pathogenic and can cause diseases in humans by producing toxins, and therefore these microorganisms are important to render harmless (Gunberg, 2008). However, beneficial bacteria include *Lactobacillus* believe to possess probiotic characteristics where it can help in improving one's health (Marteau and Shanahan, 2003)

According to WHO, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Another definition from Marteau *et al* defined probiotics as non-pathogenic microorganisms that, when ingested, exert a positive influence on host health or physiology.

They consist of bacteria (especially lactic acid bacteria and bifidobacteria), yeasts (especially *Saccharomyces*) and moulds like *Aspergillus*, and may be present either in food (especially fermented milks), food supplements or drugs. Most probiotics are bacteria (Marteau and Shanahan, 2003).

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2.2 Bacterial action on spoilage of coconut milk

Different microbiological associations between microbes and food affect the properties of the food in different ways. These associations usually cause microbiological spoilage of the food. These microorganisms can sometimes cause food spoilage or, if pathogenic, cause food borne illnesses in humans. Food borne illnesses is a major issue worldwide, not restricted to a particular country (Wareing and Fernandes, 2007).

The microbial growth in food depends on four different categories of factors. The factors are the physico-chemical properties of the food itself, conditions of the storage environment, properties and interactions of the microorganisms present and processing factors. Temperature is a crucial factor in determining the growth of microorganism as

different microorganisms have different temperatures for their optimum of growth (Jay, 2000).

The generation time for multiplication of bacteria in untreated coconut milk was found to drop from 232 min at 10°C to 44 min at 30°C (Gunberg, 2008). This leads to a rapid spoilage of the coconut milk. If the condition of storage is at a higher and at an optimum temperature for the bacteria it will then grow well.

Spoilage food like coconut milk could be caused by microbial, physical or chemical processes (Gunberg, 2008). Often spoilage of coconut milk is result of microbiological activities. These activities result in changes in the coconut milk. They include visible effects or in chemical products of microbial metabolism. Due to microbiological action in the coconut milk also lead to unpleasant smell and flavour of the coconut milk. According to Gunberg again, growth of microorganism has to be prevented and the microorganisms have to be killed in order for the food to be considered safe and can be stored.

2.3 Relationship of pH and microorganisms in food

pH is one of the intrinsic factors that affect the growth and survival of microorganisms in food. It is equal to the negative logarithm of the hydrogen activity. pH values below 7 are acidic and above 7 indicates alkaline environment (Adams, 2000). In general, microorganisms are able to grow over a wide pH range from 1.0 to 11 .0 (Padan , 1981).

Further, Adams stated that the activity and stability of macromolecules such as enzyme depends on acidity and alkalinity of an environment (Adams, 2000). Thus, the growth and metabolism of microorganism is hugely influenced by pH. Basophilic microorganism can grow well in basis environment. Meanwhile, acidic environment is suitable for acidic microorganism. Bacteria, especially pathogenic bacteria, are very sensitive to pH changes in its medium or environment (Ibrahim *et al.*, 1996).

Generally, microorganisms grow best in neutral environment because the cytoplasm pH value of living things is about 7. pH value of some food is affected by many factors thus pH range which could hold certain microorganisms is not absolute (Ibrahim *et al.*, 1996).

Coconut milk is a low-acid liquid food, with a pH of around 6.2 (Gunberg, 2008). Most bacteria grow best in the pH range 6 to 8, thus making coconut milk a suitable medium for growth of microorganisms (Jay, 2005).

Spoilage of coconut milk turns it to sour taste. This indicates the presence of acidic compound in coconut milk which reduces its pH. Lactic acid bacteria (LAB) are among widely known food-spoilage bacteria which produces acid in food (Wareing and Fernandes, 2007).

LAB has a long history of safe use in fermented foods like yoghurt and kefir. Nowadays, LAB still plays an important role in the majority of food fermentations. One of the most important contributions of these microorganisms is the extended shelf life of fermented products. Besides that, LAB also has beneficial influence on nutritional value as well as on the standardization of end products of fermented food (Oliveira *et al.*, 2008).

Overacidification of fermented products like yoghurt may result in excessive acid and flavor defects due to unrestricted growth and fermentation in yogurt (or postacidification) by the lactic culture (Hutkins and Nannen, 1993).

2.4 Preservation of coconut milk by chemicals

One of the methods to preserve coconut milk is through acidification (Gonzales, 1986). Acidification of coconut milk to a pH of 4.5 eliminates the necessity for retorting of the canned product. Acidification can be done by adding certain acids like acetic acid or citric acid. Same pH value resulted in different growth of a microorganism, if the acid used is different (Ibrahim *et al*, 1996).

2.5 Effect of pH changes on bacteria

Adjustment of pH affects bacterial growth and reproduction. In study by Shuhaimi *et al.* in 1999, *Lactobacillus acidophilus* count increases at pH 6.5 but decreases at extreme pH like pH 1, 2 and 3.

When extracellular pH (pH_{ex}) is lowered rapidly from 5 to 7, the intracellular pH (pH_i) of *Lactobacillus innocua* remained neutral (between 7 and 8) meanwhile pH_i values of all of the strains of lactic acid bacteria investigated (*Lactobacillus delbrueckii* subsp.

bulgaricus, *Streptococcus thermophilus*, and *Lactococcus lactis)* decreased to approximately 5.5 as the pH_{ex} was decreased (Siegumfeldt *et al.*, 2000).

One of the important physiological requirements of lactic acid bacteria is their ability to regulate their cytoplasmic or intracellular pH. Unlike lactic acid bacteria, other types of bacteria cells are unable to maintain a near neutral intracellular pH during growth or storage at low extracellular pH. They may lose viability and affecting the cellular activity (Hutkins and Nannen, 1993).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials and chemicals

Coconut milk, sterile distilled water, distilled water, 1M acetic acid, 1M hydrochloric acid (HCL), concentrate HCL, MacConkey agar, Blood agar, MRS agar, Triple Sugar Iron agar, Simmons Citrate agar, Methyl Red/Voges-Proskaeur medium, SIM medium, catalase reagent (3% hydrogen peroxide), coagulase plasma, oxidase reagent, normal saline, pipette (t-1000), t-1000 pipette tip, 1 ml disposable syringe, 10 ml disposable syringe, test tubes, parafilm, tin foil, test tube rack, water bath, 500 ml Scott Duran bottle, 200 Scott Duran bottle, 50 ml beaker, 100 ml beaker, 500 ml beaker, weighing boat, 10 ml measuring cylinder, 100 ml measuring cylinder, glass rod (hockey stick), Petri dish, pH meter, filter paper, wire loop, stab wire, slide, Image Analyzer (Model: BX41/CNXS), microscope Olympus B205, 37°C incubator.

3.2 Test sample

3.2.1 Coconut

Coconut used in this experiment is bought from a local vendor. The husk of the coconut is already been removed by the vendor.

3.2.2 Coconut milk extraction

The coconut is been cut open and coconut mill is used to extract the coconut milk. Only white layer of coconut milk (coconut cream) is taken to reduce risk of contamination. Coconut flesh is then put into cheese cloth and the coconut milk is extracted.

3.2.3 Coconut milk dilution

10 ml of undiluted coconut milk is pipette into a test tube. Several tests are prepared by adding 9 ml of sterile distilled water. 1 ml of undiluted coconut milk is pipette into 9 ml of sterile distilled water, making 1/10 dilution. Serial dilution is conducted till $1/10^{11}$.