NOVEL AND EFFICIENT PROTOCOL TO EXPEDITE TOTAL PLATE COUNT (TPC) AND YEAST/MOLD DETECTION FOR INDUSTRIAL SAMPLES

MISS ORANA RATTANABUMRUNG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF ENGINEERING (FOOD ENGINEERING)
FACULTY OF ENGINEERING
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Thesis Committee

…………………………………………………	Chairman of Thesis Committee
(Assoc. Prof. Suwit Siriwattanayotin, M.Eng.)	
…………………………………………………	Member and Thesis Advisor
(Asst. Prof. Aluck Thipayarat, Ph.D.)	
…………………………………………………	Member
(Assoc. Prof. Punchira Vongsawasdi, Ph.D.)	
………………………………………………	Member
(Asst. Prof. Chanan Phonprapai, Ph.D.)	

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Abstract

This research aimed to propose efficient protocols to accelerate the Total Plate Count (TPC) as well as yeast and mold detection for industrial samples. Micro Inoculation Culture (MIC), together with digital microscopy, was utilized to expedite colony detection and economically improve the analytical cost per sample. Several colony enumeration standards (i.e., Pour plate, Spread plate, and Petrifilm$^{\text{TM}}$) were validated against the MIC technique. The sensitivity and accuracy of each method were compared using pure cultures as well as industrial samples. Plate Count Agar (PCA), Chromocult$^{\circledR}$ Coliform Agar (CCA) and Potato Dextrose Agar (PDA) were used as standard media in the TPC and yeast and mold experiments. The MIC technique requires only 10 µl inoculum as opposed to 100 µl in the case of the spread plate technique and 1000 µL in the cases of the pour plate and Petrifilm$^{\text{TM}}$ techniques. The colony count results of the MIC technique were statistically comparable to those of the routine techniques. The validation of the colony counts showed good linearity with the detection limit of higher than 2 log CFU/ml. The colony count results of the frozen ready-to-eat, plasticine and dough clay samples showed similar results to those of the Pour plate technique. In addition, the effect of CCA on *Escherichia coli* colony size and chromatic development was evaluated at different incubation temperatures (i.e., 30, 35, 37, 40 and 45^{o}C). The optimal nutrient composition of Potato Dextrose Broth (PDB) can improve yeast cell multiplication and colony detection. Normally, yeast and mold growth requires 2-5 days.

However, the digital microscopy prototype was able to shorten the yeast and mold colony detection time to approximately 24 h.

Keywords: *Escherichia coli*/ Micro Inoculation Culture/ Mold/ Total Plate Count/ Yeast

หัวข้อวิทยานิพนธ์	นวัตกรรมและกระบวนการที่มีประสิทธิภาพในการวิเคราะห์จำนวนจุลินทรีย์ทั้งหมดและยีสต์/ราอย่างรวดเร็วในตัวอย่างผลิตภัณฑ์อุตสาหกรรม
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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อนำเสนอต้นแบบที่มีประสิทธิภาพในการเร่งการวิเคราะห์จำนวนจุลินทรีย์ทั้งหมดและยีสต์และราในตัวอย่างผลิตภัณฑ์อุตสาหกรรม โดยใช้เทคโนโลยีการเพาะเชื้อระดับจุลภาค ซึ่งมีกล้องจุลทรรศน์ดิจิทัลสำหรับใช้เร่งการตรวจพบโคโลนีและใช้เพิ่มความคุ้มค่าเชิงเศรษฐศาสตร์ในแง่ของราคาต้นทุนการวิเคราะห์ต่อตัวอย่าง ทั้งนี้ได้ทวนสอบเทคนิคการเพาะเชื้อระดับจุลภาคกับวิธีการทั่วไปที่ใช้ในการตรวจนับจำนวนจุลินทรีย์ได้แก่ Pour plate, Spread plate และ Petrifilm^{TM} นอกจากนี้ยังได้ทำการเปรียบเทียบความไวตลอดจนความถูกต้องของแต่ละวิธีโดยใช้เชื้อบริสุทธิ์และตัวอย่างจากทางภาคอุตสาหกรรม สำหรับอาหารเลี้ยงเชื้อแข็งที่ใช้เป็นมาตรฐานในการทดลองหาจำนวนจุลินทรีย์ทั้งหมดและยีสต์และราได้แก่ Plate Count Agar (PCA), Chromocult$^{®}$ Coliform Agar (CCA) และ Potato Dextrose Agar (PDA) ปริมาตรของตัวอย่างที่ใช้สำหรับเทคนิคการเพาะเชื้อระดับจุลภาคและเทคนิค Spread plate คือ 10 และ 100 ไมโครลิตร ตามลำดับ และเท่ากับ 1,000 ไมโครลิตร สำหรับเทคนิค Pour plate และเทคนิค Petrifilm^{TM} จากการเปรียบเทียบเชิงสถิติของผลการนับจำนวนจุลินทรีย์โดยใช้เทคนิคการเพาะเชื้อระดับจุลภาคกับเทคนิคปกติพบว่าเทคนิคทั้งสองให้ค่าที่สอดคล้องกันและมีความสัมพันธ์เป็นแบบเส้นตรง โดยจำกัดการตรวจพบเมื่อจำนวนจุลินทรีย์สูงกว่า 2 log CFU/ml ผลการตรวจนับจำนวนจุลินทรีย์ทั้งหมดในตัวอย่างอาหารแช่แข็งพร้อมรับประทานจากภาคอุตสาหกรรม ดินน้ำมัน และแป้งโดว์ พบว่าได้ผลที่คล้ายกันกับการใช้เทคนิค Pour plate นอกจากนี้ยังได้ศึกษาผลของอาหารเลี้ยงเชื้อแข็ง CCA ที่มีต่อขนาดและการสร้างสีของเชื้อ *Escherichia coli* โดยทำการเปรียบเทียบผลของอุณหภูมิเพาะบ่มที่แตกต่างกันได้แก่ 30, 35, 37, 40 และ 45 องศาเซลเซียส และยังศึกษาผลขององค์ประกอบของสารอาหารที่เหมาะสมที่สุดในอาหารเลี้ยงเชื้อเหลว Potato Dextrose Broth (PDB) ซึ่งสามารถใช้

ปรับปรุงการเพิ่มจำนวนและการตรวจพบเซลล์ยีสต์ โดยทั่วไปยีสต์และราจะต้องใช้เวลาในการตรวจพบ 2-5 วัน แต่เมื่อใช้ต้นแบบกล้องจุลทรรศน์ดิจิทัลพบว่าสามารถลดระยะเวลาการตรวจพบยีสต์และราเหลือเพียง 24 ชั่วโมง

คำสำคัญ : *Escherichia coli*/ จำนวนจุลินทรีย์ทั้งหมด/ เทคโนโลยีการเพาะเชื้อระดับจุลภาค/ ยีสต์/ รา

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CONTENTS

LIST OF TABLES

TABLE **PAGE**

TABLE **PAGE**

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

LIST OF SYMBOLS

SYMBOL

μ_{max} = Maximum Specific Growth Rate (min^{-1})

X_{max} = Maximum colony area / Maximum blue color intensity

CHAPTER 1 INTRODUCTION

1.1 Introduction

Thailand has been among the world leaders in exporting agricultural produce, ready-to-eat products and other food-related items. These export products must fulfill the international hygiene standards, such as HACCP and GMP (Atsuka et al., 2010). The maintenance of the highest standards of quality and hygiene is the key of Thailand's competitiveness to meet the world's demanding markets. The presence of pathogenic microorganisms in the export food products or the failure to detect these pathogenic contaminants may lead to a dreadful and costly effect. Although the world's food safety and export food quality have dramatically improved over the year, the advances in food manufacturing technolgy is uneven and foodborne outbreaks from microbial contamination, chemicals and toxins are still common in many part of the world (WHO, 2007).

All industrial food products must go through rigorous quality controls for sensory and microbiological analyses. There are quite a few key food-borne pathogens and microbial hygienic indicators. Among many pathogens causing frequent food-borne outbreaks, there are *Listeria monocytogrnes*, *Vibrio vulnificus*, *Salmonella typhi*, *Staphylococus aureus* and *Escherichia coli* O157:H7 (Diane, 2010; Vijayalak et al., 2010). Especially, *E. coli* O157:H7 is considered one of the most dangerous pathogens as the cell number required for infection is low (Biao, 2010). Except for the symptom of bloody diarrhea, in some cases the infection of *E. coli* O157:H7 can be complicated by hemolytic uremic syndrome, which may lead to kidney failure or death (Griffin, 1991). The consequence of the infection imposes a lot of social and financial setbacks. Also the presence of yeast/mold indicates a significant quality link of the entire food production chain because that considered a good indicator for poor hygienic condition of production sanitation. Some moulds can grow and produce mycotoxins such as *Fusarium ameniacum* produces Beauvericin while certain yeasts and moulds can cause infections or allergies (Wayne, 2012).

Conventional plating methods are mostly used for the isolation and enumeration of food-borne pathogens and yeast/mold in foods. A widely-utilized technique is the FDA official method for the mycological analysis of foods (BAM, 2001). This method requires relatively long time and substantial analyst effort for media preparation and a 5- day incubation period (Tournas et al., 2011). A serious drawback is that, although the conventional methods demand no expensive infrastructure and are rather cheap in consumables, they are laborious to perform, demand large volumes usage of and solid media and reagents, and involve time-consuming procedures both in operation and data collection. Alternative microbiological methods may help the industry to find new ways of obtaining reliable results more efficiently to ensure high standards of food safety (Vicky, 2010). The micro-scale cultivation and digital microscopy-assisted technique have been proposed to reduce the workload and facilitate the work flow by reducing the manipulations and/or the necessity for a full lab infrastructure (Saeaung, 2010; Supanivatin, 2011). This concept can be used to shorten the time to perform Total Plate Count (TPC), yeast and mold detection.

The purposes of this research were to propose the alternative method for detection of TPC, yeast/mold and perform the validation of the new technique with the standard method.

1.2 Objectives

1. Develop fast and efficient TPC, yeast and mold detection methodology to promote a high throughput industrial routine detection.

2. Explore optimal conditions for TPC, yeast and mold growth and visual improvement of colony detectability.

3. Evaluate and optimize TPC, yeast and mold enrichment to accelerate analytical routines for industrial application.

1.3 Scopes

1. To optimize growth conditions and growth parameters in micro-scale environment cultivation for achieving the highest growth of TPC, yeast and mold.

2. To improve sensitivity and effectiveness of the agar enrichment step, lower medium cost and simplify enrichment procedure for TPC, yeast and mold detection in suspended cell cultivation.

1.4 Expected Benefits

1. To shorten and simplify the conventional protocol allowing industry to perform microbiological analysis as often as possible.

2. Provide high throughput platform of rapid and efficient TPC, yeast and mold detection for safety purposes.

3. Save millions of baht worth of samples contaminate detection of finished goods being destroyed at oversea port of entry.

CHAPTER 2 THEORY AND LITERATURE REVIEWS

This chapter provides the theory and literature review related to this study including Thai food product, morphology and characteristic of *Escherichia coli*, yeast and mold traditional detection method and modern rapid method.

2.1 Thailand's Exported Food Products

Thailand is one of the world's largest producers of food products such as rice, canned tuna, frozen seafood, chicken and canned pineapple. In 2011, the value of Thai food exports increased 20% from the previous year owing to strengthened economic performance amongst major food importers such as the US, Japan, and ASEAN countries.

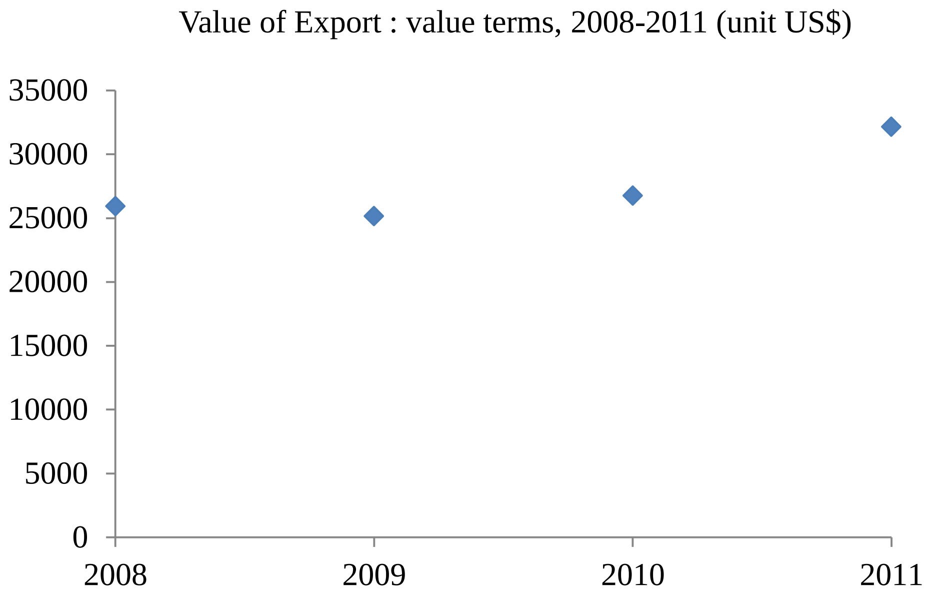


Figure 2.1 Thailand's Exported Food Products, 2008 –2011 (Source: *National Food Institute*)

Thailand's ambition to establish itself as the Kitchen of the World has dramatically increased awareness about, and focus on, safe and durable processed food. Therefore high levels of safety and quality assurances are being implemented. Even the 9000 odd cottage and small enterprises maintain the highest standards of quality and hygiene which is the key to their competitiveness and access to some of the world's most demanding markets which have stringent and exacting access standard (Murray, 2007).

2.2 Food-Borne and Waterborne Diseases

Many microorganism contaminating food and water can cause acute gastroenteritis or inflammation of the stomach and intestinal lining. When food is the source of the pathogen, the condition is often called food poising. Gastroenteritis can arise in two ways. The microorganisms may actually produce a food-borne infection. That is, they may first colonize the gastrointestinal tract and grow within in, then either invade host tissues or secrete exotoxins. Alternatively the pathogen may secrete an exotoxin that contaminates the food and is then ingested by the host. This is sometimes referred to as a food intoxication because the toxin is ingested and the presence of living microorganisms is not required. Because these toxins disrupt the functioning of the intestinal mucosa they are called enterotoxins. Common symptoms of enterotoxin poisoning are nausea, vomiting and diarrhea.

Worldwide, diarrheal diseases are second only to respiratory diseases as a cause of adult death; they are the leading cause of childhood death, and in some parts of the world they are responsible for more years of potential life lost than all other causes combined. For example, each year around 5 million children (more than 13,600 a day) die from diarrheal diseases in Asia, Africa and South America. In the United States estimates exceed 10,000 deaths per year from diarrhea and an average of 500 childhood deaths are reported.

2.3 Morphology and characteristic of Yeast & Mold

Yeasts and moulds in food product can periodically cause problems, both economic and sensory. The food especially contain high levels of sugars and other nutrients, and they possess an ideal water activity for microbial growth; their low pH makes them particularly susceptible to fungal spoilage, because a big part of the bacterial competition is eliminated since most bacteria prefer near neutral pH.

2.3.1 Yeasts

Yeasts are unicellular fungi that reproduce by budding, that is, by forming and pinching off daughter cells. (Fig 2.2) Yeast cells are much larger (about five to eight times) than bacterial cells. The best-known (and most useful) species is “bakers’ yeast,” *Saccharomyces cerevisiae*, used in bread making and in fermentations for wine and beer production.

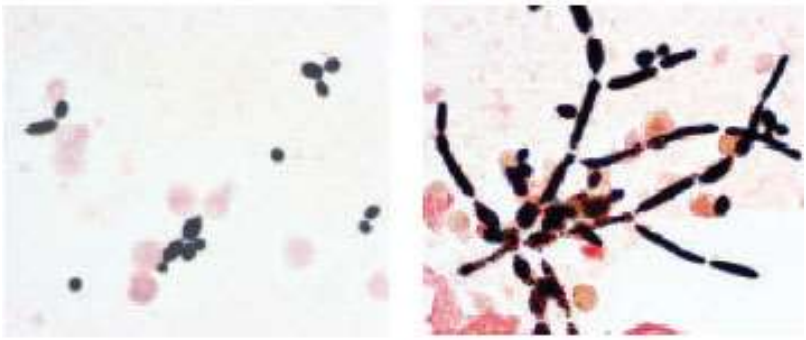


Figure 2.2 Gram stain of *Candida albicans* cells isolated from the blood culture of a patient. At left the yeast cells are budding, and at right, they have formed long, filamentous, irregularly staining hyphae

2.3.2 Mold

Molds are multicellular, higher forms of fungi. They are composed of filaments called *hyphae*, abundantly interwoven in a mat called the *mycelium*. Specialized structures for reproduction arise from the hyphae and produce *conidia* (also called *spores*), each of which can germinate to form new growth of the fungus. The visible growth of a mold often has a fuzzy appearance because the mycelium extends upward from its vegetative base of growth, thrusting specialized hyphae that bear conidia into the air. This portion is called the *aerial* mycelium. You have often seen this on moldy bread or other food, and you have probably also noted that different molds vary in color (black, green, yellow) because of their conidial pigment.

Figure 2.3 Colonies of three *Aspergillus species.* Some molds may be recognized by the color of their spores (conidia). Clockwise from left: *A. flavus* (yellow), *A. fumigatus* (smoky gray-green), *A. niger* (black)

Most of the thousands of species of yeasts and molds that are found in nature are saprophytic and incapable of causing disease. Indeed, many are extremely useful in the processing of certain foods (such as cheeses) and as a source of antimicrobial agents. *Penicillium notatum,* for example, is the mold that produces penicillin.

2.4 Impact of Yeast & Mold infection

The large and diverse group of microscopic foodborne yeasts and molds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile environmental requirements. Although the majority of yeasts and molds are obligate aerobes (require free oxygen for growth), their acid/alkaline requirement for growth is quite broad, ranging from pH 2 to above pH 9. Their temperature range (10-35°C) is also broad, with a few species capable of growth below or above this range. Moisture requirements of foodborne molds are relatively low; most species can grow at a water activity (a_w) of 0.85 or less, although yeasts generally require a higher water activity.

Both yeasts and molds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on food type, organisms involved, and degree of invasion; the contaminated food may be slightly blemished, severely blemished, or completely decomposed, with the actual growth manifested by rot spots of various sizes and colors, unsightly scabs, slime, white cottony mycelium, or highly colored sporulating mold. Abnormal flavors and odors may also be produced. Occasionally, a food appears mold-free but is found upon mycological examination to be contaminated. Contamination of foods by yeasts and molds can result in substantial economic losses to producer, processor, and consumer.

Several foodborne molds, and possibly yeasts, may also be hazardous to human or animal health because of their ability to produce toxic metabolites known as mycotoxins. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though the generating organisms may not survive food preparation, the preformed toxin may still be present. Certain foodborne molds and yeasts may also elicit allergic reactions or may cause infections. Although most foodborne fungi are not infectious, some species can cause infection, especially in immunocompromised populations, such as the aged and debilitated, HIV-infected individuals, and persons receiving chemotherapy or antibiotic treatment.

2.5 Thai FDA Revising Yeast and Mold Level in Foods

Thai Food and Drug Administration (FDA) have regulated yeast and mold in food products. Due to the no-tolerance level imposed by the Thai FDA on these contaminants, disputes between the Thai FDA and Thai food manufacturers and importers have arisen with the latter arguing that yeast and mold free food is not realistic as there are also natural or unavoidable yeast and mold in some foods that present no health hazards for humans. In addition, the weather conditions in Thailand, humid and warm, are perfect for yeast and mold to multiply. Therefore, the Thai FDA is proposing to amend its regulations to address these concerns by revising the level of yeast and mold in some food categories taking into consideration the fact that some products, even those manufactured under “Good Manufacturing Practice” guidelines, will contain certain levels of yeast and mold that don’t present a food safety risk. The revised notifications notified to the WTO are G/SPS/N/THA/191, 192, 193, 31/Rev.1, 33/Rev.1, and 34/Rev.1 on September 28-29 and target six food categories beverages in sealed containers, coffee, tea, chocolate, weight control foods, and electrolyte drinks. It is to note that the amended regulations are trade enhancing and will facilitate trade of U.S. agricultural products. The revision of level of yeast and mold found in foods would ease the import process for those six product groups, which are categorized under specifically-controlled and standardized food categories. To register those products with the Thai FDA, the importers require submitting the lab analysis result of yeast and mold. The exports of those six product groups from the US to Thailand valued over $27 million in 2009.

The proposed revised level of yeast and mold required the amendment of six MOPH notifications currently defined non-tolerance level of yeast and mold in foods as follows:

- Notification of the Ministry of Public Health No. 83 B.E. 2527 (1984) Re: Chocolate dated 15th November B.E. 2527 (1984).
- Notification of the Ministry of Public Health No.121 B.E. 2532 (1989) Re: Weight Control Food dated 23rd May B.E. 2532 (1989).
- Notification of the Ministry of Public Health No. 195 B.E. 2543 (2000) Re: Electrolyte Drinks dated 19th September B.E. 2543 (2000).
- Notification of the Ministry of Public Health No. 196 B.E. 2543 (2000) Re: Tea dated 19th September 2543 (2000).
- Notification of the Ministry of Public Health No. 197 B.E. 2543 (2000) Re: Coffee dated 19th September 2543 (2000).
- Notification of the Ministry of Public Health No. 214 B.E. 2543 (2000) Re: Beverages in Sealed Container dated 19th September 2543 (2000).

The method of analysis for yeast and mold must comply with the Bacteriological Analytical Manual (BAM) Online, U.S. Food and Drug Administration (updated version) or equivalent method. All notifications will be come into effect as from the day following date of their publication in the Government Gazette. The new proposed level of yeast and mold allowed to be found in foods are show in Table 2.1

Table 2.1 The new proposed level of yeast and mold allowed to be found in foods

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
1. Chocolate and its products	Free of mold for 1g of product	Less than 100 CFU/g
2. Coffee (Product definition can be found in the MOPH Notification No.197)		
• UHT or sterilized ready to drink coffee	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized ready to drink coffee	Free of yeast and mold	Less than 100 CFU/ml
• Ready to drink coffee in dry form dissolved as per instruction on the package	Free of yeast and mold	Less than 100 CFU/g
3. Weight Control Food		
• UHT or sterilized weight control in liquid form	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized weight control in concentration, dry, and semi-solid form	Free of yeast and mold	Less than 10 CFU/g
• Other than UHT or sterilized weight control in liquid, concentration, dry, and semi-solid form	Free of yeast and mold	Less than 100 CFU/g
4. Electrolyte Drinks		
• UHT or sterilized electrolyte drinks	Free of mold	Less than 1 CFU/ml
• Other than UHT or sterilized electrolyte drinks	Free of mold	Less than 100 CFU/ml
• Electrolyte drink in dry form dissolved as per instruction on the package	Free of mold	Less than 100 CFU/g

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
5. Beverages in Sealed Container		
• Water with dissolved Carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/ml
• Other than UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/ml
Beverages in concentrated form and needs to be diluted before consumption:		
• UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 10 CFU

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
• UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 10 CFU/g
Beverages in concentrated form and needs to be diluted before consumption:		
• Other than UHT or sterilized beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
• Other than UHT or sterilized beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
Beverages in dry form:		
• Beverages, which is containing or made from fruit, plants, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g
• Beverages, which is containing or made from other ingredients, except fruits, plant, or vegetables, and may also contain dissolved carbon dioxide or oxygen	Free of yeast and mold	Less than 100 CFU/g

Product Category	Yeast and Mold Level Allowed to be Found in food	
	Before Amendment	After Amendment
6. Tea		
• UHT or sterilized ready to drink tea	Free of yeast and mold	Less than 1 CFU/ml
• Other than UHT or sterilized ready to drink tea	Free of yeast and mold	Less than 100 CFU/ml
• Ready to drink tea in dry form dissolved as per instruction on the package	Free of yeast and mold	Less than 100 CFU/g

(Source: *MOPH Notifications on the Revised of yeast and mold Level Allowed to be Found in foods*)

2.6 Enumeration of Yeasts and Mold in Food

The dilution plating and the direct plating methods may be used to detect fungi in foods. The direct plating method is more efficient than the dilution plating method for detecting individual mold species, including most of the toxin producers, but it is less effective in detecting yeasts. It is also used to determine whether the presence of mold is due to external contamination or internal invasion.

2.6.1 Conventional Plate Count Method

The basis of methods used for the testing of yeast and mold in foods is very well established, and relies on the incorporation of a food sample into a nutrient medium in which microorganisms can replicate thus resulting in a visual indication of growth. Such methods are simple, adaptable, convenient and generally inexpensive. However, they have two drawbacks: firstly, the tests rely on the growth of organisms in media, which can take many days and result in a long test elapse time; and secondly, the methods are manually oriented and are thus labor intensive.

2.6.1.1 Spread-plate method.

Aseptically pipette 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculums with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

2.6.1.2 Pour-plate method.

Use sterile cotton-plugged pipette to place 1.0 ml portions of sample dilution into prelabeled 15 x 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered Potato Dextrose Agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

2.7 Pathogenic *Escherichia coli*

Theodor Escherichia first described *E. coli* in 1885, as Bacterium coli commune, which he isolated from the feces of newborns. It was later renamed *Escherichia coli*, and for many years the bacterium was simply considered to be a commensally organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Schulze et al., 2006).

The GI tract of most warm-blooded animals is colonized by *E. coli* within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997.

E. coli is the head of the large bacterial family, *Enterobacteriaceae*, the enteric bacteria, which are facultatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. The *Enterobacteriaceae* are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella* and *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g.*Escherichia*, *Enterobacter* and *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans.

2.7.1 Physiology

E. coli is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extra intestinal (aerobic or anaerobic) habitats.

Figure 2.4 Unstained cells of *E. coli* viewed by phase microscopy. about 1000X magnification. CDC

E. coli can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism the bacterium can survey the chemical contents in its environment in advance of synthesizing any enzymes that metabolize these compounds. It does not wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment.

2.7.2 *Escherichia coli* in the Gastrointestinal Tract

The commensal *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise no more than 1% of the total bacterial biomass. The *E. coli* flora is apparently in constant flux. One study on the distribution of different *E. coli* strains colonizing the large intestine of women during a one year period (in a hospital setting) showed that 52.1% yielded one serotype, 34.9% yielded two, 4.4% yielded three, and 0.6% yielded four. The most likely source of new serotypes of *E. coli* is acquisition by the oral route. The intestinal strains tend to displace one another about three or four times a year.

2.7.3 Pathogenesis of *E. coli*

Over 700 antigenic types (serotypes) of E. coli are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagellar antigen) is uniquely responsible for causing HUS (hemolytic uremic syndrome). Nowadays, particularly for diarrheagenic strains (those that cause diarrhea) pathogenic E. coli are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic E. coli usually requires that the isolates first be identified as E. coli before testing for virulence markers.

Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). The diseases caused (or not caused) by a particular strain of E. coli depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses. These are summarized in Table 2.2 and applied to the discussion of pathogenic strains *E. coli* below.

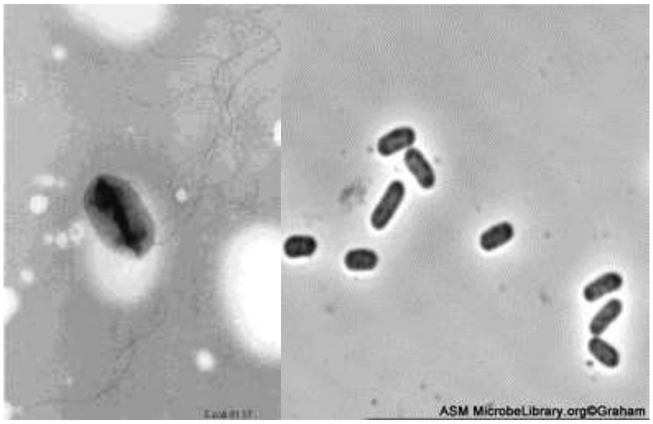


Figure 2.5 Unstained cells of *E. coli* O157:H7

2.7.4 Traveler’s Diarrhea and *Escherichia coli* Infections

E. coli may cause diarrheal disease by several mechanisms, and six categories or strains of diarrheagenic *E. coli* are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EHEC), enteropathogenic *E. coli* (EAggEC), and diffusely adhering *E. coli* (DAEC).

The enterotoxigenic *E. coli* (ETEC) strains produce one or both of two distinct enterotoxins, which are responsible for the diarrhea and distinguished by their heat stability: heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). The genes or ST and LT production and for colonization factors are usually plasmid-borne and acquired by horizontal gene transfer. ST binds to a glycoprotein receptor that is coupled to guanylate cyclase on the surface of intestinal epithelial cells. Activation of guanylate cyclase stinulates the production of cyclic guanosine monophosphate (cGMP), which leads to the secretion of electrolytes and water into the lumen of the small intestine, manifested as the watery diarrhea characteristic of an ETEC infection. LT binds to specific gangliosides on the epithelial cells and activates membrane-bound adenylate cyclase, which leads to increased production of cyclic adenoside monophosphate (cAMP) through the same mechanism employed by cholera toxin. Again, the result is hypersecretion of electrolytes and water into the intestinal lumen.

The eteroinvasive *E. coli* (EIEC) strains cause diarrhea by penetrating and multiplying within the intestinal epithelial cells. The ability to invade the pithelial cells is associated with the presence of a large plasmid; EIEC may also produce a cytotoxin and an enterotoxin.

The enteropathogenic *E. coli* (EPEC) stains attach to the brush border of intestinal epithelial cells and cause a specific type of cell damage called effacing lesions. Effacing lesions or attaching-effacing (AE) lesions represent destruction of brush border microvilli adjacent to adhering bacteria. This cell destruction leads to the subsequent diarrhea. As a result of this pathology, the ters AE *E. coli* is used to describe true EPEC strains. It is now known that AE *E. coli* is an important cause of diarrhea in children residing in developing countries.

The enterohemorrhagic *E. coli* (EHEC) strains carr the genetic determinats for attaching-effacing lesions and Shiga-like toxin production. The attaching-effacing lesion causes hemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhea. The Shiga-like toxins I and II (also called verotoxins 1 and 2) have also been implicated in two extraintestinal diseases; hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. It is belived these toxins kill vascular endothelial cells. A major form of EHEC is the *E. coli* O157:H7 that has caused many outbreaks of hemorrhagic colitis in the Unted States since it was first recognized in 1982. Currently there are a minimum of 20,000 *E. coli* O157:H7 cases and 250 deaths in the United States each year.

The enteroaggregative *E. coli* (EAggEC) strains adhere to epithelial cells in localized regions, forming clumps of bacteria with a “stacked brick” appearance. Convectional extracellular toxins have not been detected in EAggEC, but unique lesions are seen in epithelial cells, suggesting the involvement of toxins.

The diffusely adhering *E. coli* (DAEC) strains adhere over the entire surface of epithelial cells and usually cause disease in immunologically naïve or malnourished children. It has been suggested that DAEC may have an as yet undefined virulence factor.

Diagnosis of traveler's diarrhea caused by *E. coli* is based on past travel history and symptoms. Laboratory diagnosis is by isolation of the specific type of *E. coli* from feces and identification using DNA probes, the determination of virulence factors, and the polymerase chain reaction. Treatment is with fluid and electrolytes plus doxycycline and trimethroprim-sulfamethoazole. Recovery is usually without complications. Prevention and control involve avoiding contaminated food and water.

Table 2.2 Bacteria That Cause Acute Bacterial Diarrheas and Food Poisonings

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Staphylococcus aureus	1-8 (rarely, up to 18)	+++	+	Staphylococci grow in meats, dairy and bakery products and produce enterotoxins.	Enterotoxins act on receptors in gut that transmit impulse to medullary centers; may also act as superantigens.	Abrupt onset, intense vomiting for up to 24 hours, recovery in 24-48 hours. Occurs in persons eating the same food. No treatment usually necessary expect to restore fluids and electrolytes.
Bacillus cereus	2-16	+++	++	Reheated fried rice causes vomiting or diarrhea.	Enterotoxins formed in food or in gut from rowt of *B. cereus*.	With incubation period of 2-8 hours, mainly vomiting. With incubation period of 8-16 hours, mainly diarrhea.
Clostridium perfringens	8-16	±	+++	Clostridia grow in rewarmed meat dishes. Huge numbers ingested.	Enterotoxin produced duing sporulation in gut, causes hypersecretion.	Abrupt onset of profuse diarrhea; vomiting occasionally. Recovery usual without treatment in 1-4 da Many clostridia in cultures of foo and feces of patients.

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Clostridium botulinum	18-24	±	Rare	Clostridia grow in anaerobic foods and produce toxins.	Toxin absorbed from gut and blocks acetylcholine release at neuromuscular junction	Diplopia, dyshagia, dysphonia, difficulty breathing. Treatment requires clear airway, ventilation, and intravenous polyvalent antitoxin. Exotoxin present in food and serum. Mortality rate high.
Escherichia coli (enterotoxigenic strain)	24-72	±	++	Organisms grow in gut and are a major cause of traveler's diarrhea.	Heat-labile (LT) and heat-stable (ST) enterotoxins cause hypersecretion in small intestine.	Usually abrupt onset of diarrhea; vomiting rate. A serious infection in newborns. In adults, "traveler's diarrhea" is usually self-limited n 1-3 days.
Vibrio parahaemolyticus	6-96	+	++	Organisms grow in seafood and in gut and produce toxin, or invade.	Toxin causes hypersecretion; vibrios invade epithelium; stools may be bloody.	Abrupt onset3of diarrhea in groups consuming the same food, especially crabs and other seafood. Recovery is usually complete in 1-3 days. Food and stool cultures are positive.

Organism	Incubation Period (Hours)	Vomiting	Diarrhea	Epidemiology	Pathogenesis	Clinical Features
Salmonella spp. (gastroenteritis)	8-48	±	++	Organisms grow in gut.	Superficial infection of gut, little invasion. Infective dose >10^5 organisms.	Gradual or abrupt onset of diarrhea and low-grade feer. Nausea, headache, and muscle aches common. No antimicrobials usless systemic dissemination is suspected. Stool cultures are positive. Prolonged carriage is frequent.
Salmonella typhi (typhoid fever)	10-14 days	±	±	Bacteria invade the gut epithelium and reach the lymph nodws, liver, spleen, and gallbladder.	Symptoms probably due to endotoxins and tissue inflammation. Infective dose ≥10^7 organisms.	Initially fever, headache, malaise, anorexia, and muscle pains. Fever may reach 40C by the end of the first week of illness and lasts for 2 or more weeks. Diarrhea often occurs, ad abdominal pain, cough, and sore throat may be prominent. Antibiotic therapy shortens duration of the illness.

Adapted from: www.realrawmilkfacts.com

2.8 Microbial Detection Methods

Available bacterial detection methods can be divided into two groups which are traditional methods based on culturing and biochemical test and the techniques developed to shorten the detection time such as real time PCR, immunoassay or molecular technique.

2.8.1 Conventional microbiological techniques

The convectional microbiological techniques are based on the established method of incorporating food samples into nutrient media and incubating for a period of time to allow the microorganisms to grow. The detection or counting method is then a simple visual assessment of growth. These methods are thus technically simple and relatively in expensive, requiring no complex instrumentation. The methods are however very adaptable, allowing the enumeration of different groups of microorganisms.

Before testing, the food sample must be converted into a liquid from in order to allow mixing with the growth medium. This is usually done by accurately weighing the sample into a sterile container and adding a known volume of sterile diluent (the sample to diluents ratio is usually 1:10); this mixture is then homogenized using a homogenizer that breaks the sample apart, releasing any organisms into diluents. The correct choice of diluent is important. If the organisms in the sample are stressed by incorrect pH or low osmotic strength, then they could be injured or killed, thus affecting the final result obtained from the microbiological test. The diluent must be well buffered at a pH suitable for the food being tested and be osmotically balanced. When testing some food (e.g. dried products) which may contain highly stressed microorganisms, then a suitable recovery period may be required before the test commences, in order to ensure cells are not killed during the initial phase of test procedure (Davis and Jones, 1997)

The enumeration of organisms in samples is generally done by using plate count, or most probable number (MPN) methods. The former are the most widely used, whilst the latter tend to be used only for certain organisms (e.g. *Escherichia coli*) or groups (e.g. coliforms).

2.8.1.1 Plate count method

The plate count method is based on the deposition of the sample, in or on agar layer in a Petri dish. Individual organisms or small groups of organisms will occupy a discrete site in the agar, and on incubation will grow to form discrete colonies that are counted visually. Various types of agar media can be used in this form to enumerate different types of microorganisms. The use of a non-selective nutrient medium that is incubated at 30°C aerobically will result in a total viable count or mesophilic aerobic count. By changing the conditions of incubation to anaerobic, a total anaerobe count will be obtained. Altering the incubation temperature will result in changes in the type of organism capable of growth, thus showing some of the flexibility in the convectional agar approach. If there is a requirement to enumerate a specific type of organism from the sample, then in most cases the composition of the medium will need to be adjusted to allow only that particular organism to grow. There are three approaches used in media design that allow a specific medium to be produced: the elective, selective and differential procedures.

Elective procedures refer to the inclusion in the medium of reagents, or the use of growth conditions, that encourage the development of the target organisms, but do not inhibit the growth of other microorganisms. Such reagents may be sugars, amino acids or other growth factors. Selective procedures refer to the inclusion of reagent or the use of growth conditions that inhibit the development of non-target microorganisms. It should be noted that, in many cases, selective agents will also have a negative effect on the growth of the target microorganism, but this will be less great than the effect on non-target cells. Example of selective procedures would be the inclusion of antibiotics in a medium or the use of anaerobic growth conditions. Finally, differential procedures allow organisms to be distinguished from each other by the reactions that their colonies cause in the medium. An example would be the inclusion of a pH indicator in a medium to differentiate acid –

producing organisms. In most cases, media will utilise a multiple approach system, containing elective, selective and differential components in order to ensure that the user can identify and count the target organism.

The types of agar currently available are far too numerous to list. For details of these, the manuals of media manufacturing companies (e.g. Oxoid, LabM, Difco and Merck) should be consulted.

2.8.1.2 MPN method

The second enumerative procedure, which is the Most Probable Number (MPN) Method or a Durham Test. In principle, this Durham Test relies on the lactose fermentable characteristic (APHA, 1992). The Most Probable Number (MPN) Method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed and then uses the combinations of positive results to consult a statistical tables, to estimate the number of organisms present. Typically only the first 2 phases are performed in coliforms and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. The 5-tube MPN is used for water, shellfish and shellfish harvest water testing and there is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (APHA, 1998).

As indicated earlier, this method is used only for particular types of test and tends to be more labour and materials intensive than plate count methods. In addition, the confidence limits are large even if many replicates are studied at each dilution level. Thus the method tends to be less accurate than plate counting methods but has the advantage of greater sensitivity.

2.8.1.2.1 MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g food into sterile high-speed blender jar. Frozen samples can be softened by storing it for ≤18 h at 2-5°C, but do not thaw. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades. Prepare decimal dilutions with sterile Butterfield's phosphate diluent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm or vortex mix for 7 s. Do not use pipette to deliver <10% of their total volume. Transfer 1 mL portions to 3 LST tubes for each dilution for at least 3 consecutive dilutions. Hold pipette at angle so that its lower edge rests against the tube. Let pipette drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

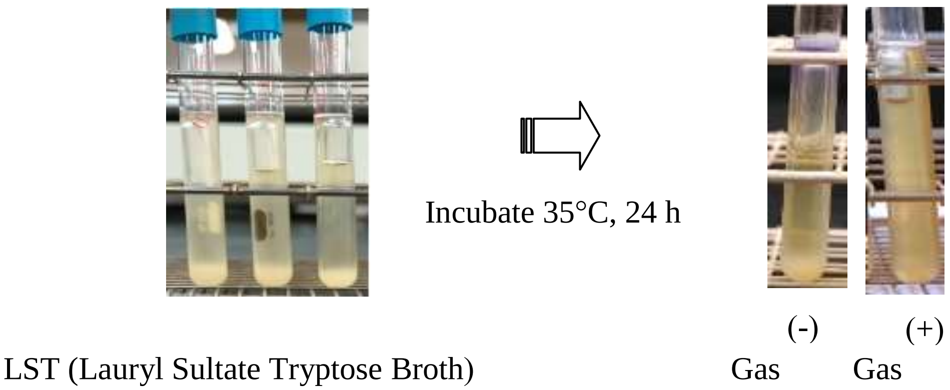


Figure 2.6 Presumptive tests for coliforms, fecal coliforms and *E. coli*

Incubate LST tubes at 35°C. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

2.8.1.2.2 MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35°C, because most coliforms grow at 35°C (Weiss et al., 1983) and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) of coliforms based on proportion of **confirmed** gassing LST tubes for 3 consecutive dilutions.

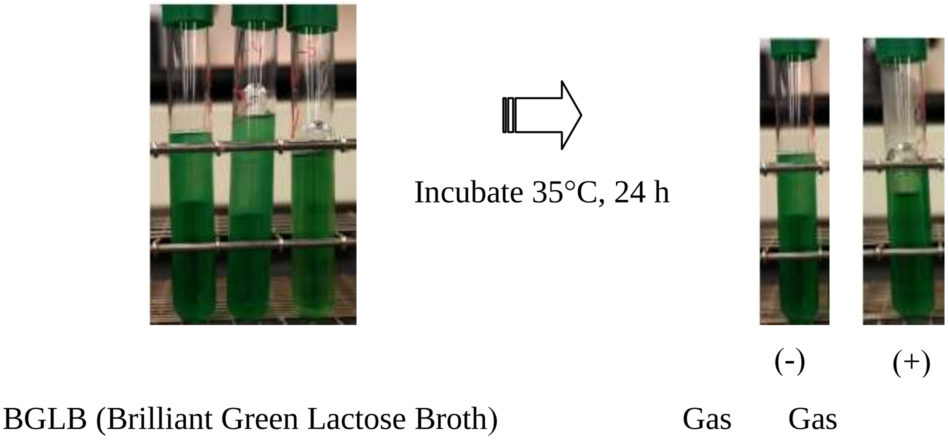


Figure 2.7 Confirmed test for coliforms

2.8.1.2.3 MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). EC medium was chosen because : the medium contains bile salts, which inhibit most spore – forming or gram – positive bacteria capable of fermenting lactose. (Warren et al., 1978) Incubate EC tubes 24 ± 2 h at 45.5 °C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis.

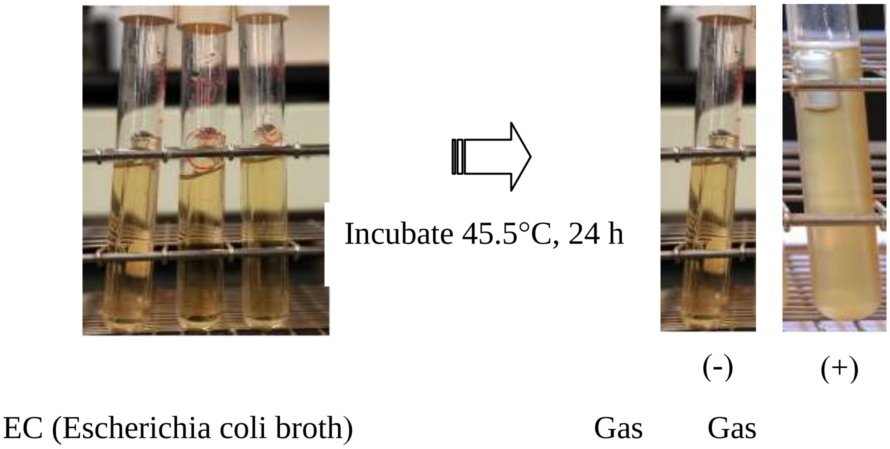


Figure 2.8 Confirmed test for fecal coliforms and *E. coli*

2.8.1.2.4 MPN - Completed test for *E. coli*.

To perform the completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to **5** suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35°C and use for further testing.

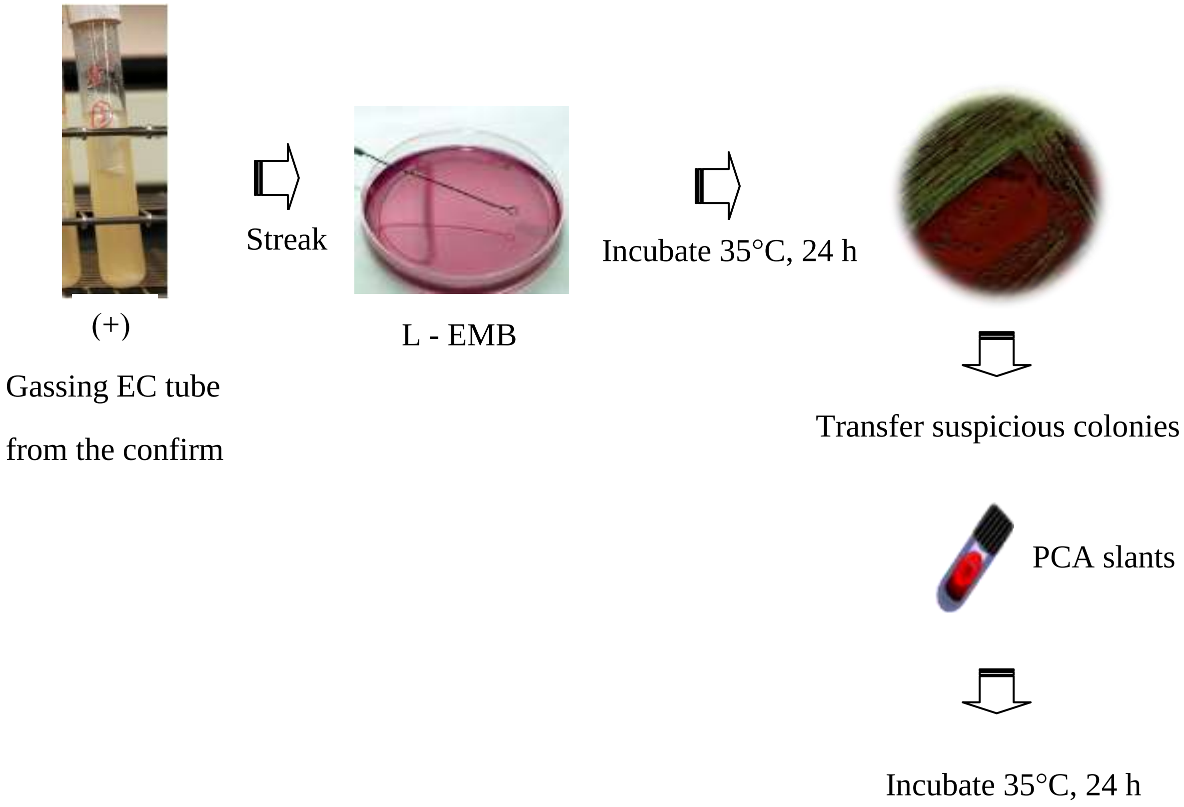


Figure 2.9 Completed test for *E. coli*

2.8.2 The Modern Rapid Method of Yeast & Mold *and Escherichia coli* Detection

2.8.2.1 The Enzyme – based Method

New techniques have been developed recently for detection and differentiation of bacteria. They are based on the utilization of chromogenic and/ fluorogenic substrates for detection of activities of specific enzymes. Theses sensitive methods have led to improved accuracy and faster detection and may be performed by using the microbiological growth media that contain enzyme substrates linked to a chromogen (colour reaction), fluorogen (fluorescent reaction) or a combination of both. The target population is characterized by enzyme systems that metabolize the substrate (sugar or amino acid) to release the chromogen/fluorogen. This results in a colour change in the medium and/or fluorescence under long wave UV light. The incorporation of such fluorogenic or chromogenic enzyme substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain micro-organisms. (Schonenbrucher et al., 2008 : Perry and Freydiere, 2007 : Greenwood et al., 2005 and Manafi, 1996, 2000)

The Chromogenic enzyme substrates are compounds which act as the substrate for specific enzymes and change colour due to the action of the enzyme. In general, based on their chemical reaction, four groups of chromogenic compounds can be distinguished and recently described by Manafi (1998). Indolyl derivatives are water soluble and heat stable and the mostly used derivatives such as 5-bromo-4-chloro-3-indolyl (X), 5-bromo-6-chloro-3-indolyl (magenta) or 6-chloro-3- indolyl (salmon) showing no diffusion on the agar plate.

The Fluorogenic enzyme substrates generally consist of a specific substrate for the specific enzyme such as sugar or amino acid and a fluorogen such as 4-methylumbelliferone, being able to convert UV light to visible light. Methylumbelliferyl-substrates are water soluble, highly sensitive and very specific. Because of their pH-dependance, strong diffusion in solid media and the need of UV - light, the use of these substrates is limited.

Figure 2.10 Chromogenic culture media products available for the isolation and identification of yeasts and mold in food products

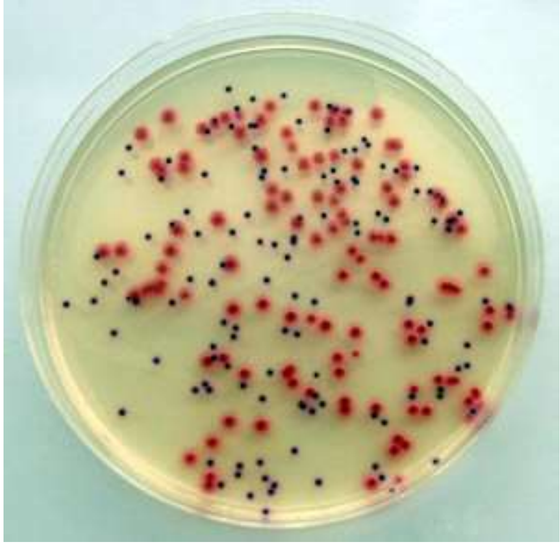


Figure 2.11 *E. coli*/coliform Selective Agar is a chromogenic medium for the detection and enumeration of *E. coli* and other coliforms (important hygiene indicators) from food and water samples. *E. coli* colonies turn a distinctive cherry red color whereas coliforms colonies turn blue

2.8.2.2 Rapid and automated methods

The general interest in alternative microbiological methods has been stimulated in part by the increased output of food production sites. This has resulted in the following greater numbers of samples being stored prior to positive release- a reduction in analysis time would reduce storage and warehousing costs. A greater sample throughput being required in laboratories- the only way that this can be achieved is by increased laboratory size and staff levels, or by using more rapid and automated method. A requirement for a longer shelf-life in the chilled foods sector- a reduction in analysis time could expedite product release thus increasing the shelf-life of the product. The increased application of HACCP procedures- rapid methods can be used in HACCP verification procedures.

Table 2.3 Pros and cons of conventional and rapid methods in bacterial detection

Conventional methods	**Rapid methods**
- Lower cost compared with molecular tools	- Fast detection
- Time consuming	- Time consuming
- Labor intensive	- Expensive cost/analysis

There are a number of different techniques referred to as rapid methods and most have little in common either with each other or with the convectional procedures that they replace. The methods can generally be divided into quantitative and qualitative tests, the former giving a measurement of the number of organisms in a sample, the latter indicating only presence or absence. Laboratories considering the use of rapid methods for routine testing must carefully consider their own requirements before purchasing such a system. Every new method will be unique, giving a slightly different result, in a different timescale with varying levels of automation and sample throughput. In addition, some methods may work poorly with certain types of food or may not be able to detect the specific organism or group that is required. All of these points must be considered before a method is adopted by a laboratory. It is also of importance to ensure that staff using new methods are aware of the principles of operation of the techniques and thus have the ability to troubleshoot if the method clearly shows erroneous results.

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial Strains

E. coli (from Department of Medical Science; DMST, Thailand)
S. cerevisiae (from Department of Medical Science; DMST, Thailand)
A. niger (from Department of Medical Science; DMST, Thailand)

3.2 Equipments and Instruments

1. Laminar flow cabinet, DWYER Series 0325, USA
2. Refrigerated centrifuge, Hitachi 35S I, Japan
3. Hot air incubator, Memmert Model ULM500, Japan
4. 96-microwell plate, Costar, USA.
5. Aluminum pot
6. Autoclave, BECTHAI and HIRAYAMA Model HA300D, Japan
7. Auto pipette volume 10 microlitter, Autopipette, USA
8. Auto pipette volume 200 microlitter, Autopipette, USA
9. Auto pipette volume 1000 microlitter, Autopipette, USA
10. Auto pipette volume 5 milliliter, Autopipette, USA
11. Balance accuracy 0.0001 grams, Metter Toledo Model AG204, Switzerland
12. Balance accuracy 0.01 grams, Metter Toledo Model GG4002-S, Switzerland

3.3 Media and Chemical Reagent

1. Non-Selective media
    1.1 Plate count agar (PCA), Didco, USA
    1.2 Trypticase soy agar (TSA), Difco, USA
    1.3 Trypticase soy broth (TSB), Difco, USA
    1.4 Potato dextrose agar (PDA), Difco, USA
    1.5 Potato dextrose broth (PDB), Difco, USA
2. Selective media

2.1 Chromocult® coliform agar (CCA), Difco, USA

3. Sodium chloride (NaCl), Merck, Germany

3.4 Experimental Design

The designed experiment was completely random. The data were investigated using the analysis of variance (ANOVA), a statistical program SPSS version 16 was used to perform the calculation. Independent variables were cultivation parameters, 2 main types including, physical parameters; initial cell concentrations, cultivation volume and nutritional parameters; medium types (both non-selective and alternative media), medium concentrations, ratios of TSB and alternative medium. Dependent variables were optical density growth curve of *E. coli*, *S.cerevisiae*, *A.niger* and growth characteristics (e.g., maximum specific growth rate: μ_{max} and the first derivative maximum of the function: X_{max}). To observed difference between factor levels, Duncan’s multiple comparisons were selected. Mean values were considered at 95% confidential interval ($\alpha=0.05$). All pair wise were compared at significant level $P<0.05$.

3.5 Sample Preparation

3.5.1 Frozen Stock of *E. coli*

Single colony of *E. coli* was grown in TSB (100 ml) and incubated at 35±2 ^{o}C (200 rpm, 8 hr) (Hayashi and Yamasaki, 1998; Karoonuthaisiri et al., 2009).

Single colony of Yeast was grown in PDB (100 ml) and incubated at 30 ^{o}C (200 rpm, overnight) (Frengova Gl, 2003).

Single colony of Mold was grown in TSB (100 ml) and incubated at 30 ^{o}C (150 rpm, 48 hr) (Adrian Tsang et al., 2009).

3.5.2 *E. coli,* Yeast and Mold Strain Preparation

E. coli culture was prepared in shake tubes using Tryptic Soy Broth (TSB), For yeast/mold using Potato Dextrose Broth (PDB) and incubated to reach the final cell density at 10^9 CFU/ml. Serial dilution was done to achieve the desired initial cell concentration at 10^4 CFU/ml. The strain of *E. coli* was confirmed and enumerated in Chromocult® Coliform Agar (CCA) using the micro-inoculation technique (MIT) as described elsewhere (Saeaung and Boonyaprapasorn, 2010 : Sangadkit et al., 2010 ;Supanivatin et al., 2010 and Khueankhancharoen et al., 2010). Different preparation strategies (i.e. Autoclave, Normal and Blender treatments) were then investigated and the *E. coli* growth kinetics was captured using a sigmoidal mathematical model.

3.5.3 Media Preparation

Vary Standard Medium Concentration

Standard media concentration (CCA) was desired 0.1X - 1X of typical concentration. The volume of 0.1X media concentration will be prepared for 100 ml and then diluted by decreasing media from 26.5 grams to 2.65 grams for prepare 0.2X, 0.3X, 0.4X, 0.5X, 0.6X, 0.7X, 0.8X, and 0.9X of CCA, respectively.

Sample Preparation Procedures

Chicken was extracted by using high temperature that are 121°C for 15 or 60 minutes by using autoclave and classical boiling at 95°C for 60 minutes as well as mushroom extraction.

Vary Alternative Media Types

All alternative media were extracted by using autoclave at 121°C for 15 minutes. There are 2 samples to cultivated Yeast and Mold (e.g. chicken, mushroom and yeast extract) (Figure 3.1) for 100 ml per each recipe. Then pipette 10 µl of desired Yeast, Mold concentration into sample extracted to test colonies count. These samples were pipette 10 µl into 24-microwell plate per hole

(a) Chicken (b) Mushroom (c) Yeast extract

Figure 3.1 All types of raw materials to prepare alternative media for yeast/mold growth

3.5.4 Chromocult® Coliform Agar Preparation

The CCA powder was mixed vigorously with distilled water in a blenderizer. The mixing was very brief and lasted 30 sec. The CCA mixture was transferred into a 1 liter media bottle and then heated using a full-power microwave setting for 2 min. Boiling during fast microwave (MW) pasteurization allowed the mixture to dissolve completely and achieve homogeneous consistency.

3.5.5 Potato Dextrose Agar Preparation

The PDA solution was prepared as described in procedure. The PDA was transferred into a 1 liter media bottle and autoclaved at 121°C (15 lbs pressure) for 15 min.

3.5.6 Cultivated Conditions Preparation

The experiments were performed using 24-microwell plate (Figure 3.2). Alternative media which interested cultivation volume were dispensed into micro well prior to inoculating *E. coli,* Yeast and Mold culture. If the cultivation volume was 200 µl, media would be pipetted into the well at 100 µl. *E. coli,* yeast and mold suspension at different initial cell concentrations 50 µl were inoculated into media. One experiment condition was performed 2 replicates.

Figure 3.2 24-microwell plate format

3.6 *E. coli*, Yeast and Mold Cell Enumeration

3.6.1 Standard Plate Count

Pour Plate Technique

Samples at a proper dilution (1 ml) were pipetted onto the plate of Plate Count Agar (PCA), then homogeneously mixed with melting agar, and incubated at 35±2ºC for 48 hr. Plates of those dilutions yielding 25– 250 colonies were enumerated.

Spread Plate Technique

0.1 ml of liquefied sample at a proper dilution was pipetted onto the pre-fabricated plate of PCA, and then used five sterile glass beads to disperse the sample until the sample was dried out, and incubated at 35±2ºC for 24 hr. Plates of those dilutions yielding 20–200 colonies were enumerated.

Micro Inoculation Culture Technique

Cultivation volumes were fixed at 10 µl onto the top of PCA agar surface and incubated at 35±2ºC. Then later normally 12 – 15 hr will be detected and captured using a reflected light microscope equipped with a 1.5 megapixel (Figure 3.3). A constructed prototype of digital image analysis protocol was implemented to evaluate the area that each colony occupied on the agar surface. The experimental assumption was that the colony only expanded horizontally and the area of expansion was highly correlated with the growth of pathogens on the solid medium.

Figure 3.3 Micro Inoculation Technology Scope

3.7 Growth Characteristic Determination Using Logistic Model

The logistic mathematical representation was selected to model the *E. coli* batch growth curve. Several authors suggested to utilize this logistic function (Eq. 3.1) simulated sigmoidal-type growth profiles as shown below (Mitchell et al., 2004 and Saeaung and Boonyaprapasorn, 2010).

3.7.1 Maximum Specific Growth Rate (μ_{max})

Calculation procedures are as follow. Optical density data were saved as text file were opened by using Microsoft excel 2003 program. Data were collected in one work sheet and sorted by cultivation condition (e.g., media types). Cultivation time data and optical density growth data of *E. coli* were transferred to SigmaPlot 10.0 program to estimate μ_{max}. Growth curves were created and curve fitting were applied. Maximum specific growth rate were fixed into original built-in Sigmoid 4 Parameter (Equation 3.1).

$$y = y_0 + \frac{a}{1 + e^{-(\frac{x - x_0}{b})}} \tag{3.1}$$

y_0 = the amount of initial inoculation of *E. coli*

a = maximal value of *E. coli* growth

x_0 = the first derivative maximum of the function

b = slope of curve

μ_{max} = maximum specific growth rate, $\frac{1}{b}$ (h^{-1})

Curve Fit command would mathematically solve using real observed data, such that calculated $\frac{1}{b}$ (μ_{max}) might represent actual value reflecting experimental value.

3.8 Statistical analysis

All CFU counts per ml or gram were converted to log10 counts before statistical analysis. For each plate method combination, a paired t-test was performed on the differences in the average log10 counts between each of the two methods compare pour plate with spread plate and MIC.

3.9 Swabbing Production Line Equipment

Today’s world food manufacturing dictates that hygiene standards has to be closely monitored. It is essential to determine the cleanliness of food processing equipment and manufacturing areas before they are used for food production. Swabbing technique is commonly used to evaluate surface cleanliness, to ensure food contact surfaces are clean. It can be can be used in almost all environments, including food, medical, sports, toilets, and washrooms areas.

3.9.1 Swabbing procedure

The swabs used for sampling surfaces for microbial contamination are examples of devices that are simple in design and construction. In this research, samples collected from surfaces by specialized swabbing materials. The absorbent fabrics or the cotton tipped swabs (Figure 3.4) are moistened with sterilized water inside aseptic plastic bag. The water can be applied to large swabbing areas in the production line. Simply take the inside swabbing materials out aseptically, hold on to the fabric and then swab it on a designated surface. Take the swabbed material back into the plastic pouch and keep it always from other possible cross contamination. And then after swabbing the tested area, five milliliters of buffer solution were added, and all the contents inside the swabbing pouch were mixed thoroughly. In the laboratory, 10 μl of mixture was dropped into agar medium (e.g., Petri dish, 96-microwell , or 24-microwell)

Figure 3.4 Swabbing test kit

CHAPTER 4 RESULTS AND DISCUSSION

This chapter aimed to study and describe the development of micro-scale cultivation and digital microscopy-assisted technique to perform Total Plate Count (TPC), yeast and mold detection. For the TPC experiments, *E. coli* was utilized as a pathogen model. The enumeration of *E. coli* was performed on Plate Count Agar (PCA) and Chromocult® Coliform Agar (CCA) media and that of yeast and mold were done on Potato Dextrose Agar (PDA) medium. This research included the growth kinetics study of *E. coli,* yeast and mold in micro-scale cultivation (i.e., liquid and solid state culture media) to provide fundamental knowledge and facilitate the development of small-volume analysis.

4.1 Development of TPC enumeration concept

4.1.1 Development of detection protocol

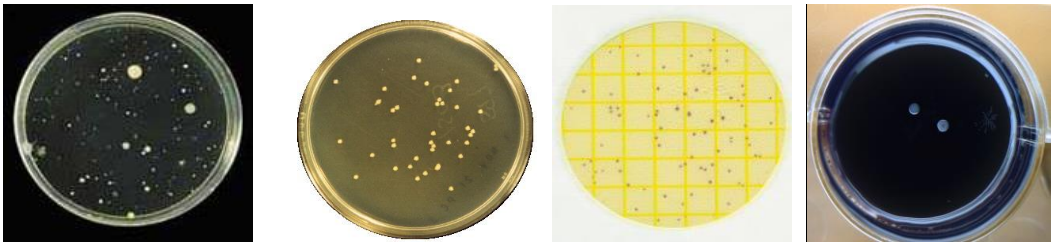
Experimental Description

The development of the Micro Inoculation Culture (MIC) was performed to compare TPC procedures including pour plate, spread plate and Petrifilms™. The microbiological aspects of methodologies were critically analyzed to reduce analytical cost and time that related the growing colony (Figure 4.1).

Results and Discussion

All of the protocols were investigated. The cultivation volumes were reduced from 1 ml (for the pour plate and Petrifilm™ techniques) and 0.1 ml (for the spread plate technique) in Table 4.1 to 0.01 ml for the MIC technique. Fung and Kraft (1968) already explored miniaturized viable cell count using 0.025 ml inoculum samples on calibrated loop. This inoculum size was still 2.5-fold more comparing to the 0.01 ml inoculum volume that used in this research. For the medium usage in step of preparation both pour plate and spread techniques utilize up to 15-20 ml of media. In contrast, the MIC technique only uses 2-3 ml of media per sample. This strategy produces 10-fold reductions in term of media use.

The pour plate and the Petrifilm™ technique demands no less than one day to perform TPC, generally 2 days required (APHA, 2001). The early detection by microscopy sets the MIC apart from the spread plate in terms of analytical time since the spread plate generally called for 1 day to be able to detect visually. However, an application of visual aid (e.g., the developed prototype digital image equipment) can further lessen the detection time owing to higher magnification power using a series of magnifying lenses (described in 4.1.2) which normally requires 12-15 h. When considering the analytical cost per sample, the Petrifilm™ technique is rather expensive to analyze industrial samples in large volume.



(a) (b) (c) (d)

Figure 4.1 Bacterial analytical techniques of BAM standard (a) pour plate (b) spread plate (c) Petrifilm™ and (d) MIC

Table 4.1 Detail comparisons of different TPC procedures including pour plate, spread plate and MIC techniques

Techniques	Pour plate	Spread plate	Petrifilm™	MIC
Inoculum size	1 ml	0.1 ml	1 ml	0.01 ml
Medium usage	15-20 ml	15-20 ml	-	2-3 ml
Counting method	Visually detection	Visually detection	Visually detection	Digital image microscope
Incubation time	2 days	1 day	2 days	12-15 h

4.1.2 Prototype equipment of colony image acquisition

Experimental Description

The development of the MIC technique in the previous topic necessitates a construction of a microbial detection prototype. Different types of optical and digital microscopes were utilized as an assisting equipment to detect the area of growing colonies at their early stage of incubation. Other lighting equipment (e.g., background lighting, LED ring, etc.) was tested for find proper lighting gear for the prototype equipment. A X-Y stage was included to move the culture plate along X and Y planes for references. The prototype of digital image acquisition system was then tested to improve the usability and clarity of the acquired colony image.

Results and Discussion

Digital microscope is an important apparatus to assist the use of MIC technique. A digital microscope with the appropriate magnification can produce good colony image in order to accelerate the detection of colony culture and improve the overall analysis. The microscope used in this experiment has a USB connection to a personal computer; hence, it is very convenient to use and provide good connectivity. This prototype was constructed to accommodate the use of 24-well plates as well as 96-well plates shown in Figure 3.2. This linear platform was used to move the microtiter plate along an X-Y plane and mounted on the base of the microscope. The focus of colony image can be adjusted using fine focus knob and sliding the microscope body along the vertical axis (Figure 4.2). Moreover, the magnification power can be altered by the zoom lenses, which can reduce the detection time due to its ability to see the colonies when they are still very small. The image was illuminated by an LED light source which has polarizable to achieve the high resolution of colony image. (Supanivatin, 2010).

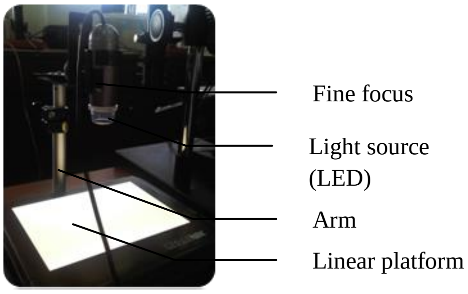


Figure 4.2 Photographs of the MIC equipment used to perform miniaturized TPC experiment

4.1.3 Validation of TPC detection protocols

Experimental Description

E. coli culture was prepared at 10^9 CFU/ml overnight prior to the enumeration experiment. The culture was serially diluted to prepare the standard cultures at different final cell densities at 10^3, 10^4, 10^5, and 10^6 CFU/ml. Then the actual cell count was performed using the micro-scale cultivation, spread plate and pour plate technique (see Chapter 3). Before carrying out the cell enumeration, the standard cultures were serially diluted 10-fold for at least 3 dilutions. The inoculum sizes were varied from 0.01 ml for MIC (in 24-well microtitter plates), 0.1 ml for spread plate (in Petri dishes), and 1 ml for pour plate techniques (in Petri dishes). All total plate count techniques were incubated at 35±2°C for 24h. For MIC, the forming colonies were observed using low magnification digital microscopy. For conventional TPC techniques, the colony count was performed using human visual detection. The number of colonies were plotted and compared between different techniques.

Results and Discussion

The conventional pour plate widely recommended by most microbiological protocol handbooks was used to validate other alternatives (Scotter, 2000). The standard plots comparing the TPC counts using different techniques was compiled in Figure 4.3. Essentially, all techniques returned practically same readings pending that the sample inoculum was prepared with proper dilution for colony evaluation. All standard slopes were close to unity with fairly good correlation coefficients.

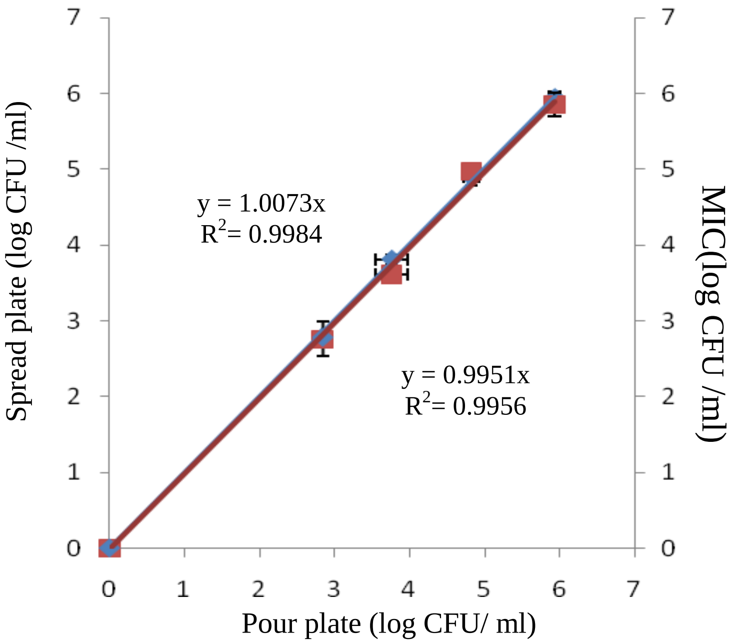


Figure 4.3 Correlation between estimates of TPC obtained by Pour- Spread plate (△) and Pour plate- MIC (X)

The images of TPC colonies in Figure 4.4 were taken at the same incubation time (approximately 24 h). However, the colony size morphology varied significantly depending on the magnification of the microscope and the intrinsic growth of TPC from different format of cultivation. Clearly, the regular protocol possesses a large agar surface to cover and allows only low-magnification image acquisition to cover the entire plate see Figure 4.4a. The spread plate facilitates higher magnification power of microscope and the colony size was enlarged significantly see Figure 4.4b. The availability of better oxidative environment for microbial growth in the spread plate not only shortened the incubation to one day but also provided more distinct colony size and appearance for easier detection. In Figure 4.4c, the TPC using the MIC strategy resulted in the well-defined and easily-to-detect colonies and sacrificed only 10 times less medium used. This new scheme of fast detection utilized only limited resources and medium is well suit all requirements for a routine industrial TPC protocol (i.e., high throughput capability, rapid response, low cost per test unit, etc.). Only with higher magnification that the differences of colony characteristics were observed and this finding would be irrelevant for human visual inspection.

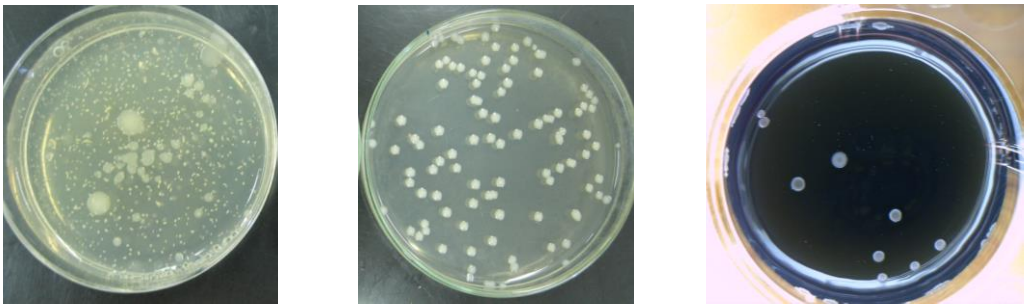

(a) **(b)** **(c)**

Figure 4.4 Photographs of colony formed on PCA using different techniques (a) pour plate, (b) spread plate and (c) MIC

4.1.4 Enhancement of plate count agar technique

Experimental Description

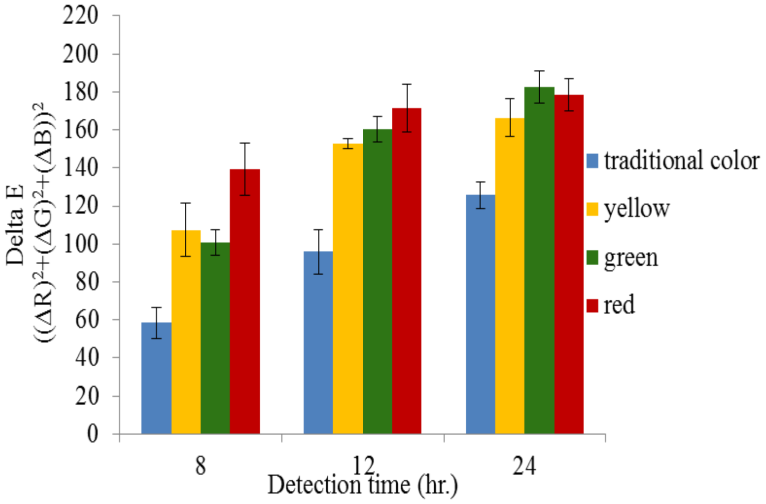
PCA mixture was prepared at 500 ml and mixed with 6.25 ml of different dye pigments (i.e., green, red and yellow colors). The PCA solution was autoclaved at 121^{o}C for 15 min and formed culture plates for *E. coli* cultivation. The incubation was maintained at 37^{o}C for 24 h. The size and characteristic of *E. coli* colonies was observed using the prototype system of image analysis. The *E. coli* colony image was digitized every hour using a reflected light microscope equipped with a 1.3 megapixel camera in. A constructed prototype of digital image analysis system was performed to evaluate the colony count.

Results and Discussion

The overall impact of different background colors of the medium on the total number of colony count was first evaluated. In Table 4.2 showed statistically the same number of total colony reading.

Table 4.2 Comparison of the total cell count of *E. coli* at different background color of the medium using plate count agar (PCA)

No. of *E. coli* count (log CFU/ml)			
Control	Green dye	Red dye	Yellow dye
9.35^{a} ±0.59	9.45^{a} ±0.38	9.48^{a} ±0.29	9.39^{a} ±0.19



[a] values in a row with different superscripts are significantly difference at $P<0.05$.

Figure 4.5 Contrast difference between colony and background color at different culture period using plate count agar (PCA)

Agar color of the medium analyzed the contrast of the colony and the background using different agar colors in RGB system. The contrast represents by delta E which is the rout R-square error of R, G and B indices (Figure 4.5). At 8 hours the delta E of the original agar color only had the differences of the color between colony and agar itself, the contrast grew up to almost twice as much. The improvement of the contrast was the same at 24 hours. This experiment the delta E of the traditional agar color was less than immediately of the adjusted agar color meaning better colony detection. The red background provided the best color contrast over 60% larger delta E value compare to the traditional agar color.

With proper background color in Figure 4.6, the maximum contrast between the medium and colony colors was very useful to improve the visual detection of *E. coli* colonies. The high efficacy and precision of colony enumeration scheme was improved by using appropriate magnification power of the digital microscope. The numbers of colonies counts from different background colors were essentially the same. The red scheme, on the other hand, was more effective in detecting *E. coli* colonies, hence, the detection time was able to be substantially reduced from overnight to 8 hours and the colony image can be kept for future reference, which is every useful for industrial applications. This fast colony count capability can enhance the quality assurance and quality control practices and ensure the highest food safety policy for local Thai food manufacturers.

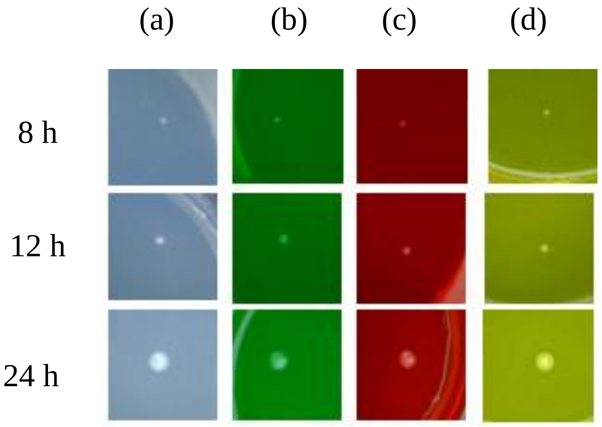


Figure 4.6 Photographs of colony formed on plate count agar using different background color of the medium (a) control; (b) green dye pigment; (c) red dye pigment and (d) yellow dye pigment

4.1.5 Implementation of TPC enumeration on industrial samples

Experimental description

The three TPC strategies were examined (i.e., pour plate, spread plate and MIC techniques) (described in 3.6.1) and implemented to food samples, including frozen ready-to-eat products and samples from production lines. For statistical assessment, the approximation of Colony Forming Units (CFU) was transformed to log CFU/ml and the statistical differences were calculated by using Tukey test.

Results and Discussion

4.1.5.1 Finished product samples

The finished product samples are critical samples for food industry. Usually, the potential contaminant was diluted and the sensitivity of detection was crucial to perform colony count for these samples. In this research, the pour plate and spread plate techniques were compared to the MIC techniques to verify the methods for industrial samples. As we concluded in the protocol validation experiment (described in 4.1.3), the implementation of pour plate, spread plate and MIC techniques did not alter the final colony count results of pure culture samples in Figure 4.4. The validation of the techniques was performed one more time on the real food product samples see in Table 4.3. All techniques had the same final colony count results. The growth of colonies, which favored oxidative pathways (the spread plate and MIC techniques), had higher total plate count readings (APHA, 2001; Buck and Cleverdon, 1960; Hoben and Somasegaran, 1982) than the pour plate technique. However, some of the reading results were insignificantly different in term of statistics.

Table 4.3 TPC enumeration in food samples using various techniques (pour plate, spread plate and MIC techniques)

Samples Type	Sample No.	Total Plate Count (log CFU/ml)		
		Pour plate	Spread plate	MIC
Somtum	1-5	ND^a	ND^a	ND^a
	6-9	TNTC^a	TNTC^a	TNTC^a
	10	$2.39^a \pm 0.02$	$2.42^b \pm 0.01$	$2.48^b \pm 0.01$
	11	$2.75^a \pm 0.04$	$2.85^a \pm 0.05$	$2.72^a \pm 0.09$
	12	$3.06^b \pm 0.02$	$3.15^b \pm 0.07$	$3.12^a \pm 0.12$
	13	$2.79^a \pm 0.04$	$2.85^a \pm 0.04$	$2.88^a \pm 0.03$
	14	$2.47^a \pm 0.04$	$2.55^b \pm 0.14$	$2.57^b \pm 0.11$
	15	$2.83^a \pm 0.08$	$2.89^a \pm 0.02$	$2.89^a \pm 0.02$
Rice	1-11	ND^a	ND^a	ND^a
	12	$2.83^a \pm 0.09$	$2.88^a \pm 0.14$	$2.89^a \pm 0.13$
	13	$2.62^a \pm 0.04$	$2.69^a \pm 0.05$	$2.67^a \pm 0.07$
	14	$2.55^a \pm 0.14$	$2.59^a \pm 0.01$	$2.52^a \pm 0.12$
	15	$2.65^a \pm 0.04$	$2.63^a \pm 0.03$	$2.69^a \pm 0.01$
Topping	1	ND^a	ND^a	ND^a
	2	ND^a	ND^a	ND^a
	3	ND^a	ND^a	ND^a
	4	ND^a	ND^a	ND^a
	5	ND^a	ND^a	ND^a

^{a,b} values in a row with different superscripts are significantly difference at $P<0.05$.

ND = Not Detect

TNTC = Too Numerous To Count

In Table 4.3, all results from three techniques agreed well. The Not Detect (ND) results in the somtum samples no.1-5, rice samples no.1-11 and topping samples no.1-5, were confirmed regardless of the difference in the inoculum volume (1 ml in the pour plate technique, 0.1 ml in the spread plate technique and 0.01 ml in the MIC technique). In general, the higher inoculum size should provide better sensitivity since more samples were included in the testing protocol (APHA, 2001). For the somtum samples no.6-9, the numbers of colonies of all techniques were Too Numerous To Count (TNTC) that is the drawback of these plating techniques if the dilution of the sample was not appropriate.

4.1.5.2 Samples from the production lines (processing swab samples)

The process and equipment must be frequently cleaned in order to reduce the pathogenic contamination in the final products. Moreover, a large number of swab samples obtained from different production lines and processing equipment were evaluated.

Table 4.4 TPC enumeration in production lines using various techniques (pour plate, spread plate and MIC techniques)

Samples	Total Plate Count (log CFU/ml)		
	Pour plate	Spread plate	MIC
Plastic curtain	ND^{a}	ND^{a}	ND^{a}
Worker gloves	ND^{a}	ND^{a}	ND^{a}
Worker hands	ND^{a}	ND^{a}	ND^{a}
Tray before freezing	TNTC^{a}	4.32^{b}±0.10	4.32^{b}±0.01
Stainless steel tray	2.54^{a}±0.14	2.95^{b}±0.14	3.11^{b}±0.34

^{a,b} values in a row with different superscripts are significantly difference at $P<0.05$.

ND = Not Detect

In Table 4.4, the first three samples (plastic curtain, worker gloves and worker hands) were aseptic. All techniques showed no colonies on TSA. If there are colonies grown on these media, the spread plate and MIC always showed higher colony count (Table 4.4 and 4.5). Similarly, the last two samples (tray before freezing and stainless steel tray) produced higher colony count in the spread plate and MIC techniques. Noted that the pour plate technique was sensitive to sample dilution. In the tray before freezing sample, the pour plate reading returned TNTC whereas the spread plate and the MIC produced countable colonies.

4.2 Protocol improvement to detect industrial hygienic microbial contaminant

4.2.1 Effect of temperature on TPC growth

The growth *E. coli* colony and its chromatic development were captured and monitored digitally using a medium-magnification microscope prototype. A mathematical model was utilized to simulate colony area expansion. The effect of temperature was to compare the *E. coli* colony growth characteristic, namely the colony area expansion and color development, on Chromocult® Coliform Agar (CCA) applying different degrees of thermal stresses. Several key kinetic parameters, including maximum specific growth rate, and the characteristic time were extracted and used to describe colony growth at different temperatures.

Experimental Description

E. coli cells were cultured in Tryptic Soy Broth (TSB) and prepared to reach the final cell density at approximately 10^9 CFU/ml. Appropriate serial dilution at 10^8 CFU/ml was achieved enabling well-distributed colony separation on Petri dishes and good resolution of digital imagery using a medium magnification microscope. The strain of *E. coli* was confirmed and enumerated in Chromocult® Coliform Agar (CCA) using spread plate technique as described elsewhere (Sangadkit et al., 2010). Different incubation temperatures (i.e., 30, 35, 37, 40 and 45°C) were then implemented and *E. coli* growth kinetics was captured using a sigmoidal mathematical model.

Results and Discussion

The overall impact of different incubation temperatures on the total number of colony count was first evaluated (Table 4.5). At high temperature (45°C), no *E. coli* cell survived on CCA. At the temperature between 30-40°C, the incubation temperature had no impact of the total colony count. Different temperature treatments returned statistically the same number of total colony reading on Chromocult® Coliform Agar (CCA). However, the same range of incubation temperature had rather significant effect on colony growth kinetics as reported in the subsequent topic where the expansions of colony areas were logged as a function of incubation time. On the macroscopic level, the effect of incubation temperature was hardly noticed.

Table 4.5 Comparison of the total cell count of *E. coli* at different incubation temperatures using Chromocult® Coliform Agar

No. of *E. coli* count (log CFU/ ml)				
Incubation temperatures				
30°C	35°C	37°C	40°C	45°C
8.35[a] ±0.05	8.35[a] ±0.06	8.44[a] ±0.05	8.50[a] ±0.03	ND

[a] values in a row with different superscripts aresignificantly difference at $P<0.05$.

ND: not detected

Despite the same total cell number from different temperature treatment the kinetics of colony area expansion at these different incubation conditions did, however, significantly differ (Figure 4.7). The use of this local *E. coli* phenotype defined a rather high incubation temperature to achieve maximum colony growth. The common incubation setting adopted from generic protocols was shown to delay the detection of *E. coli* on Chromocult® Coliform Agar. The optimal temperature setting seemed to matter when the reduction of analytical time was desirable. For instance, the use of micro inoculation culture (MIC) proposed by a research group at King Mongkut's University of Technology Thonburi (KMUTT) required fast colony inspection and early detection using a digital microscope to hasten colony enumeration and *E. coli* detection (Orenga et al., 2009 : Manafi, 1996). For conventional agar cultures (e.g., pour plate technique), the acceleration of colony expansion was irrelevant since lead time for human visual detection was a time consuming process and generally required overnight incubation.

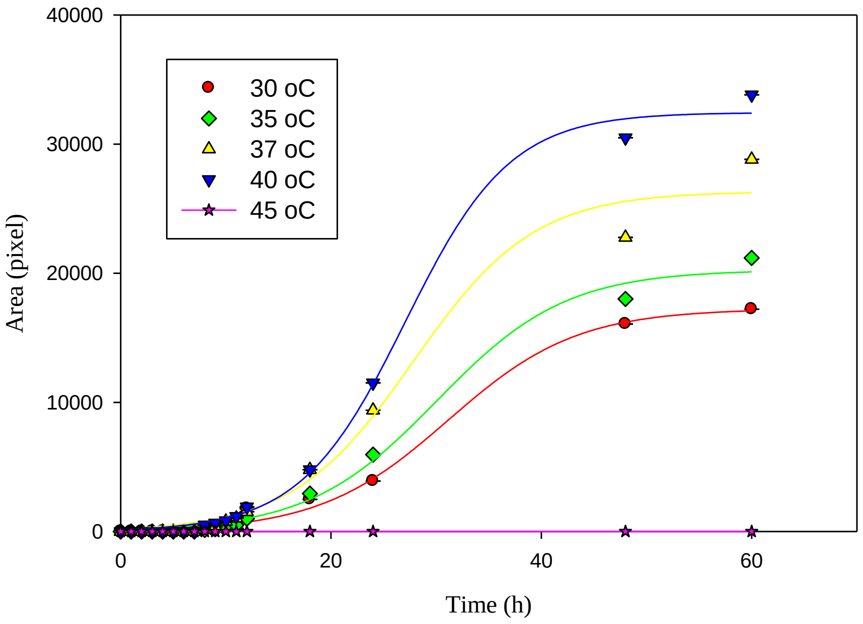


Figure 4.7 Profiles of colony growth in five different incubation conditions (red line: 30°C; green line: 35°C; yellow line: 37°C; blue line: 40°C and pink line: 45°C)

4.2.2 Kinetics of *E. coli* colony expansion

Experimental Description

There exists an optimal range of temperatures where *E. coli* colony enlarged at the highest rate and reached significantly larger final colony area. Many literatures suggested the optimal temperatures were in a rather lower temperature spectrum 37±2°C. The growth characteristics of *E. coli* in this experiment indicated the optimal range should be on a higher temperature span. The *E. coli* growth using the temperature less than 37°C substantially diminished the intrinsic growth kinetics. At low incubation temperature (e.g., 30°C), the colony did not only expand at a much slower rate but also grew to substantially smaller colonies. The incubation temperature played an important role in deteriorating or supporting colony growth of *E. coli* on Chromocult® Coliform Agar.

Results and Discussion

As seen in the colony growth profiles in Figure 4.7, the optimum incubating temperature was in the proximity of approximately 40°C. At this temperature, the maximum specific growth rate and final maximum colony area extracted from the logistic model showed the largest values (i.e., 0.200±0.002 h^{-1} and 32441±470 pixels, respectively) as shown in Table 4.6.

Table 4.6 Growth kinetics of *E. coli* on Chromocult® Coliform Agar and relative coefficient of logistic model at different incubation temperatures

	Growth kinetic parameters		
Incubation temperature (°C)	μ_{max}	X_{max}	R^2
30	0.163^{a}±0.002	17236±100	0.994±0.002
35	0.163^{a}±0.002	20256±375	0.993±0.002
37	0.174^{b}±0.002	26324±456	0.984±0.001
40	0.200^{c}±0.002	32441±461	0.997±0.001
45	ND	ND	ND

^{a,b,c} values in a column with different superscripts are significantly difference at $P<0.05$.
ND: not detected

Detection of a specific enzyme or enzymes has been implemented to differentiate and identify genus, species, or groups of microorganisms. In this case, the growth of *E. coli* colonies on chromogenic substrates involves the production of unique enzymes producing distinct dark blue to violet color to *E. coli* colonies. The β-D glucuronidase cleaves both substrates Salmon-Gal and X-glucuronide in CCA developing more intense dark blue color as the incubation time progresses (Figure 4.8) (Manafi , 1996 : Manafi, 2000 : Manafi et al., 1991). As early as 8 h, small violet colonies were detected.

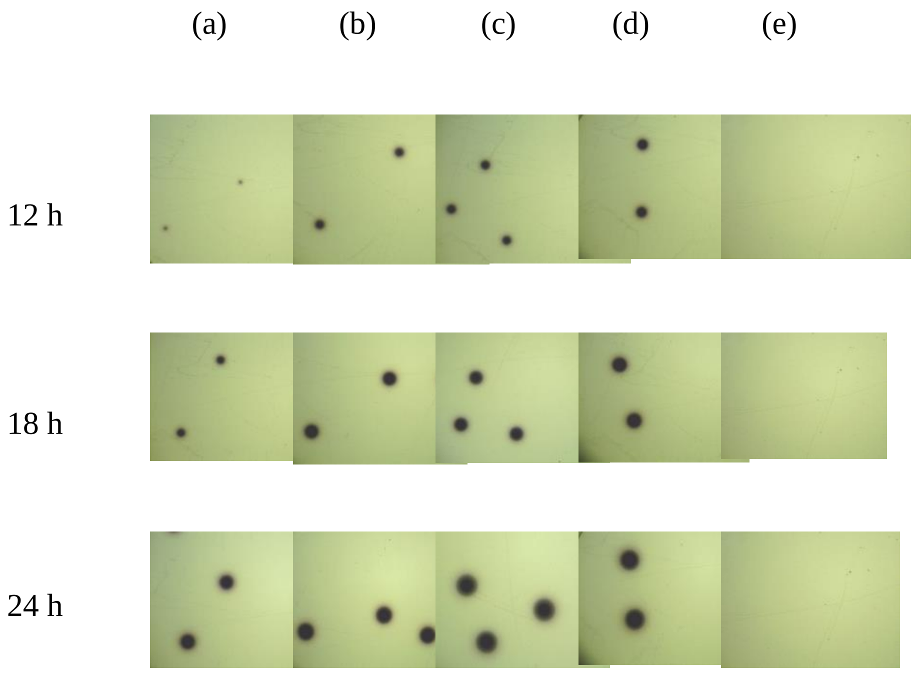


Figure 4.8 Photographs of colony formed on plate count agar using different incubation conditions (a) 30°C; (b) 35°C; (c) 37°C; (d) 40°C and (e) 45°C

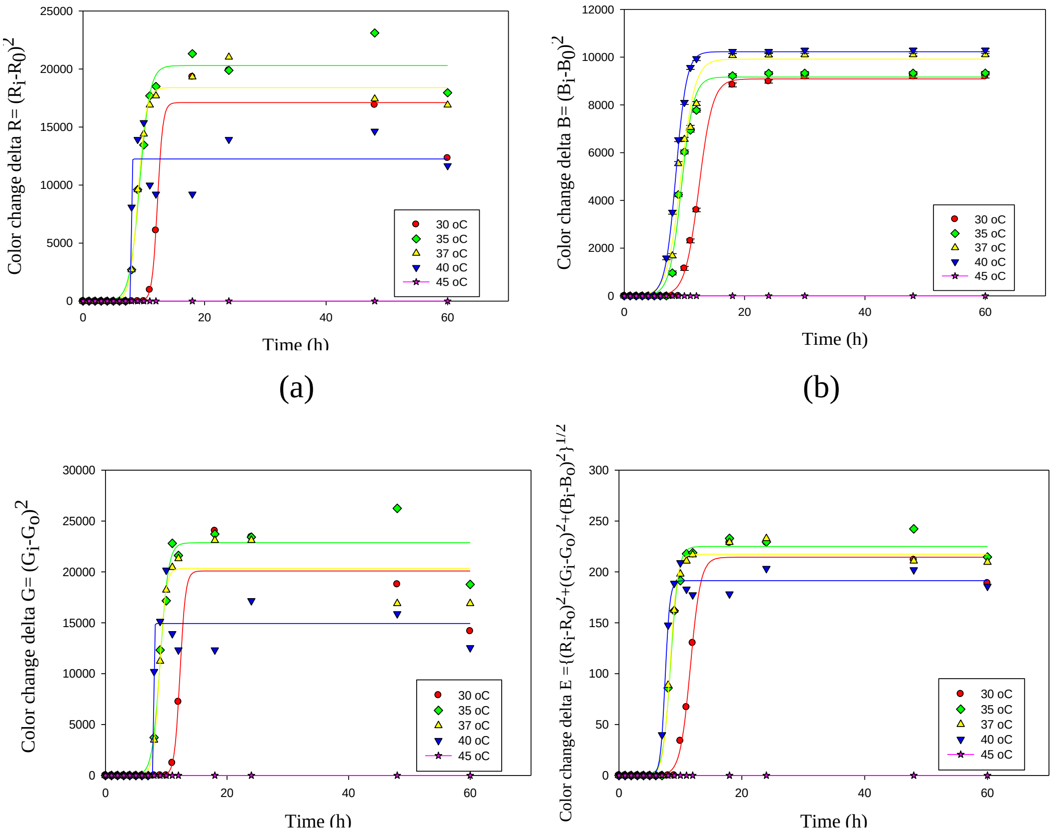
4.2.3 Kinetics of *E. coli* color development

Experimental Description

The colonies grew larger and the color was more imbued in violet chroma. The development of color kinetics was captured by the colony image showing the increment of RGB color indices contrasted to the yellowish background of the CCA (Figure 4.9). The differences between the color indices and the background color also followed sigmoidal function. Once the colony was identified, the specific color attribute of *E. coli* was able to be quickly evaluated.

Results and Discussion

The effect of incubation temperature on the color derivatives was not quite apparent (Figure 4.9). It was largely dependent on the specific attributes (R, G or B indices) and each attribute displayed different optimal incubation to facilitate color formation. Having the prior knowledge of how color of *E. coli* colony progress to dark blue chroma from X-glucuronide, the B attributes would be naturally a good candidate to represent *E. coli* colony color development. The profile of the delta B attribute in Figure 4.9b seemed to agree well with the colony growth profile (Figure 4.9) in terms of the effect of incubation temperature. The delta E representing the overall color change was rather insensitive with temperature change (Figure 4.9d). In Table 4.7, the evaluation of delta B development kinetics using the logistic model indicate the use of higher incubation temperature (around 40°C rather than 35-37°C) to expedite blue chroma development. Both maximum specific B attribute development rate and final blue chroma were maximized at the 40°C incubation treatment. The advantage of fast colony and color development kinetics was only benefit a new scheme of colony detection of medium magnification microscopy instead of human visual inspection in the conventional Petri dish format.



(a) (b)

(c) (d)

Figure 4.9 Profiles of color change using different incubation temperatures (a) delta R; (b) delta B; (c) delta G and (d) overall color change or delta E

Table 4.7 Comparison of key kinetic parameters in term of color development (delta blue)

Growth kinetic Parameter			
Incubation temperature (°C)	μ_{max}	x_{max}	R^2
30	0.868±0.027	9087.750±75.378	0.998±0.003
35	1.002±0.019	9166.750±29.691	0.991±0.002
37	0.860±0.059	9915.500±27.258	0.983±0.001
40	1.119±0.014	10226.750±95.136	0.998±0.001
45	ND	ND	ND

^{a,b,c} values in a column with different superscripts are significantly difference at $P<0.05$.
ND: not detected

The study of *E. coli* colony growth on CCA was facilitated by the use of a prototype digital imagery system. The highest colony expansion on CCA was obtained at a significantly higher incubation temperature setting (40°C) than typical setting (35°C) recommended by most microbiological standards and handbooks. The development of blue color attribute also followed the kinetics of colony area growth. The use of medium magnification digital microscopy allow the detection of both colony and blue chroma within 8 hours suggesting the detection time of *E. coli* colony can be substantially reduced from overnight with human visual detection to 8 hours by microscopy-assisted inspection. The logistic model was very useful to extract important kinetic parameters of *E. coli* colony expansion; hence, it allowed more qualitative comparison between treatment that macroscopically seemed insensitive and trivial.

4.2.4 Effect of substrate dilution and nutrient limitation on *E. coli* growth

Experimental Description

Explored the cost reduction strategy to further dilute the CCA strength and observe the effect of nutrient limitation on *E. coli* growth. Different CCA dilutions (i.e., 30, 40, 50, 60, 70, 80, 90% of CCA recipe and original CCA) were prepared to cultivate *E. coli* inoculum (approximately 10^2 CFU/ml). Colony numbers and colony color were investigated. All cultures were incubated at the same condition (35±2°C). The effect of CCA dilutions on colony count and blue pigment development were only observed when the concentration was diluted lower than 30% of the manufacturer-recommended strength. At original CCA, colony was spotted after 8 h of incubation and the dark blue color attribute was developed after 12 h. Not only lower CCA dilution affected the final colony count, but also retarded chromatic progression of *E. coli* colony on CCA.

Results and Discussion

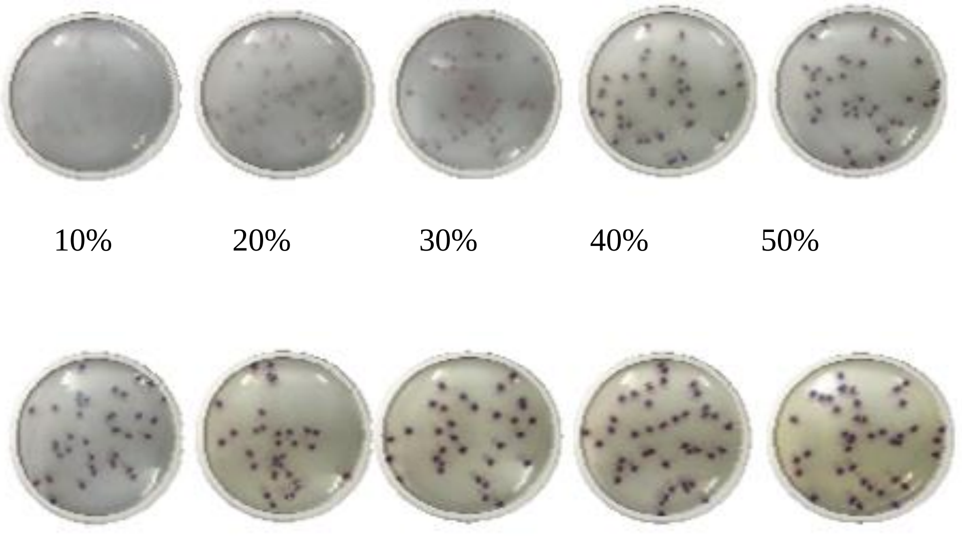
Dilution of CCA concentration produced profound effects on *E. coli* detection. The colony count result showed significantly less colony count was obtained when the concentration of CCA went beyond 30% of the original recipe (Table 4.8). At lower CAA concentrations, *E. coli* growth was restricted as the result of the combination of limited chromogenic substrate and nutrient availabilities (Turner et al., 2000). Also, the key substrates (salmon-GAL and X-glucuronide) for chromogenic reaction were diluted as the result of CCA serial dilution.

Table 4.8 Colony count on culture plates using different concentrations of CCA comparing to the original concentration

CCA dilution	*E. coli* count (log CFU/ ml)
10 %	9.11 ± 0.04^a
20 %	9.22 ± 0.04^b
30 %	9.26 ± 0.06^c
40 %	9.29 ± 0.03^d
50 %	9.28 ± 0.03^d
60 %	9.30 ± 0.01^d
70 %	9.29 ± 0.03^d
80 %	9.31 ± 0.01^d
90 %	9.30 ± 0.02^d
Original conc.	9.30 ± 0.01^d

[a,b,c,d] values in a column with different superscripts are significantly difference at $P<0.05$.

In addition to the *E. coli* count, the captured image of *E. coli* colonies displayed the subtle effect of nutrient and indicator restrictions (Figure 4.10). Noticeably, the size and purplish *E. coli* colonies were gradually changed as a result of the CCA dilution. At lower dilutions, the images of the *E. coli* colonies were very faint affecting colony numbers and the distinction of colony itself. For colony count purposes, the CCA preparation according to manufacturer's recommendation was able to reduce to 60-70% without compromising the colony detectivity by human vistualization. This result was implied that the key substrate components in the typical CCA media were present in excess for routine *E. coli* growth (Bredie et al., 1992).



10% 20% 30% 40% 50%

60% 70% 80% 90% Typical conc.

Figure 4.10 Digitized images of *E. coli* colony from pure culture after 12 h of incubation at 35±2°C

Further image analysis revealed that with the application of digital microscope and proper magnification power one could reduce the concentration of CCA to only 40% of manufacturer's recommendation (Figure 4.11). The tracking of the RGB color attributes as a function of culturing time indicated that the blue color attributes developed from beta -glucuronidase activity only differed when the CCA concentration went below

40%. The use of the developed digital microscope was able to extent the dilution further and minimizes the concentration of the CCA medium.

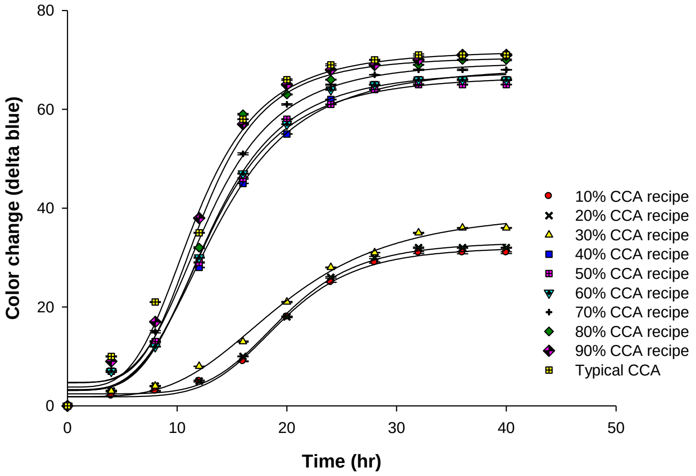


Figure 4.11 Profiles of color change (delta blue) of *E. coli* colonies as a result of varying CCA concentrations

4.3 Improvement of industrial yeast/mold detection

Potato dextrose agar (PDA) and potato dextrose broth (PDB) are common yeast/mold growth media made from potato infusion, and dextrose. They are added to many media formulations providing protein and carbohydrate sources that cells can easily access for energy production and growth. Optimal composition of nutrients can significantly enhance amino acid metabolism and aerobic respiration; hence, suitable growth media can improve cell multiplication and colony detection.

4.3.1 Optimization of liquid substrate for yeast/mold

4.3.1.1 Effect of carbon source on yeast/mold growth

Experimental Description

The typical composition of the Potato dextrose broth (PDB) composes of 200 grams of potato infusion, 20 grams of D-glucose and 1 liter of purified water (Difco^{TM} Potato Dextrose Agar). This experiment investigated the potato infusion component, which was replaced by different local sources of carbohydrates (for example, long grain rice flour, the corn flour, the cassava flour, the wheat flour, the glutinous rice flour) and compared with the actual grains (long grain rice and glutinous rice). All these carbohydrate alternatives were compared with the PDB control.

Results and Discussion

Polysaccharide substitute experiment

For the carbon (C) sources, the D-glucose is the monosaccharide that provides a major source of the maintenance energy for living organisms. The addition of potato infusion provides the source of amylose and amylopectin that can be effectively utilized by yeast/mold. Basically yeast and mold have the enzyme β-D-glucoamylase to digest the polysaccharide to monosaccharide. There is an abundance of other starch-degrading and -related enzymes in fungi as well. For example, the fungal enzyme (glucoamylase 1 or G1; 1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3) from *Aspergillus niger*, hydrolyses α-D-glucosidic bonds of starch and other polysaccharides to yield β-D-glucose (Kay et al., 1997). Therefore, yeast and mold should be able to assimilate other sources of carbohydrates, including local starch and flour that are prevailing in the country. These carbohydrates are easily accessible and less expensive comparing to those imported sources.

Table 4.9 showed the varying effect of different carbon sources used in this experiment and summarized the colony count of yeast and mold grown on these alternative carbohydrates. This table also returned statistically comparison of yeast and mold counts. Essentially, the source of carbohydrates did not alter the resulting colony count comparing to the conventional PDB control at the 95% confident level. The different carbon sources didn’t compromise the effectiveness of medium to supply adequate nutrients for yeast and mold colonies to grow.

Table 4.9 Comparison of alternative media for the enumeration of yeast/mold when perform on pure culture

No. of yeast/mold count (log CFU/ml)		
Treatments	Yeast	Mold
Long grain rice flour	5.30^{a} ±0.06	5.18^{a} ±0.09
Corn flour	5.18^{a} ±0.08	5.26^{a} ±0.10
Cassava flour	5.21^{a} ±0.01	4.95^{a} ±0.13
Wheat flour	5.51^{a} ±0.10	5.20^{a} ±0.04
Glutinous rice flour	5.20^{a} ±0.13	5.22^{a} ±0.17
Long grain rice	5.19^{a} ±0.09	5.26^{a} ±0.05
Glutinous rice	5.20^{a} ±0.09	5.08^{a} ±0.10
PDB control	5.53^{a} ±0.07	5.34^{a} ±0.13

[a] values in a column with different superscripts are significantly difference at $P<0.05$.

Not only the modified carbohydrate recipes returned the same number of colony readings, the growth kinetics (Figure 4.12 and 4.13) of the yeast and mold in this experiment demonstrated the similar growth profiles as well. In these experiments, the initial cell inoculation was started out relatively low, approximately 10^{2} log CFU/ml. The growth profile in Figure 4.12 elucidated the classic lag, log and stationary phases whereas at 10^{3} log CFU/ml initial cell concentration the mold experiments entered the exponential growth immediately. Both yeast and mold cultures approached the stationary phase around 24 h after incubation.

After 24 h, the final concentrations of yeast and mold reached about the same values as the PDB (Figure 4.12 and 4.13). There were no statistical differences between these viable cell counts. The growth kinetics was pretty much the same. Tukey test was implemented to differentiate the statistical differences. All modified carbohydrate recipes displayed no significant difference for both yeast and mold experiments.

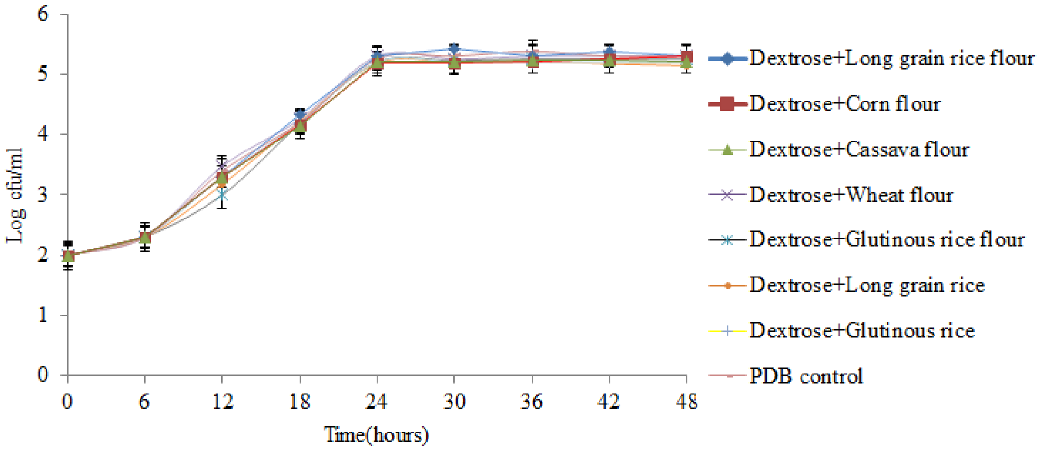


Figure 4.12 Comparison of growth models fitted to variable count data of yeast grown at 30^{o} C between PDB and alternative media

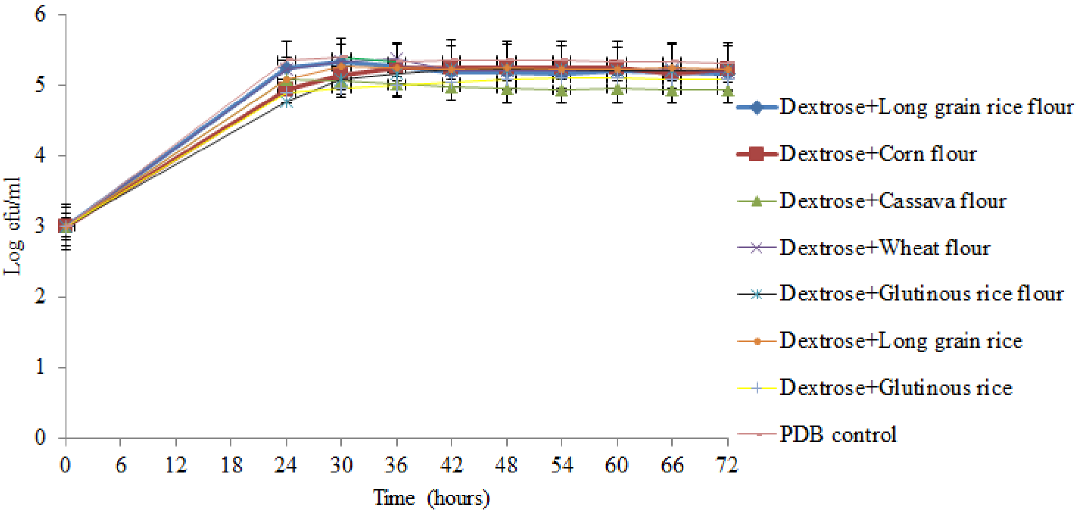


Figure 4.13 Comparison of growth models fitted to variable count data of mold grown at 30^{o} C between PDB and alternative media

Effect of monosaccharide

This experiment only uses the PDB monosaccharide (i.e., dextrose) and skips the other carbohydrate source (i.e., potato infusion). Basically it consists of just dextrose and water. D-glucose, commercially known as dextrose, is a monosaccharide (basic unit of carbohydrates, $C_6H_{12}0_6$) that is readily-absorbed carbon source of the quick energy

(Markande, 2012). This carbon source was compared with the PDB control. Tukey test was implemented to differentiate the statistical differences (Table 4.10).

Table 4.10 Viable cell counts of yeast and mold comparing pure dextrose to PDB control media using pure cultures

No. of yeast/mold count (log CFU/ml)		
Treatment	Yeast	Mold
Water + dextrose	5.76^{a} ±0.17	5.55^{a} ±0.12
PDB control	5.30^{b} ±0.21	5.32^{a} ±0.11

^{a,b} values in a column with different superscripts are significantly difference at P<0.05.

Figure 4.14 and 4.15 showed batch growth curves of yeast and mold on dextrose and water, respectively. The yeast growth is better on dextrose solution than that on PDB. Generally, dextrose is a monosaccharide that is more readily absorbed into the cell than the complex molecules (Jianzheng et. al., 2008), like carbohydrates. The growth kinetic is even better than the PDB control treatment. For the mold the growth kinetic is the same as the PDB control.

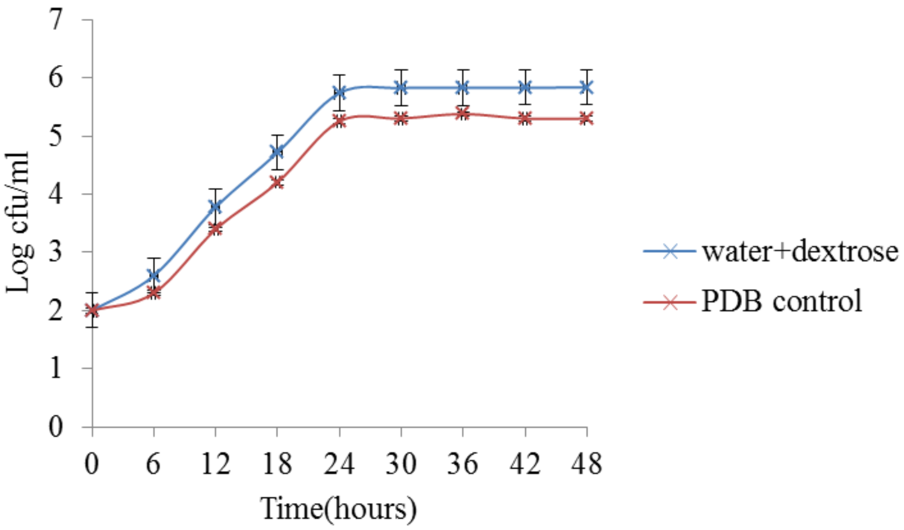


Figure 4.14 Comparison of growth models fitted to variable count data of yeast grown at 30^{o} C between PDB and alternative media

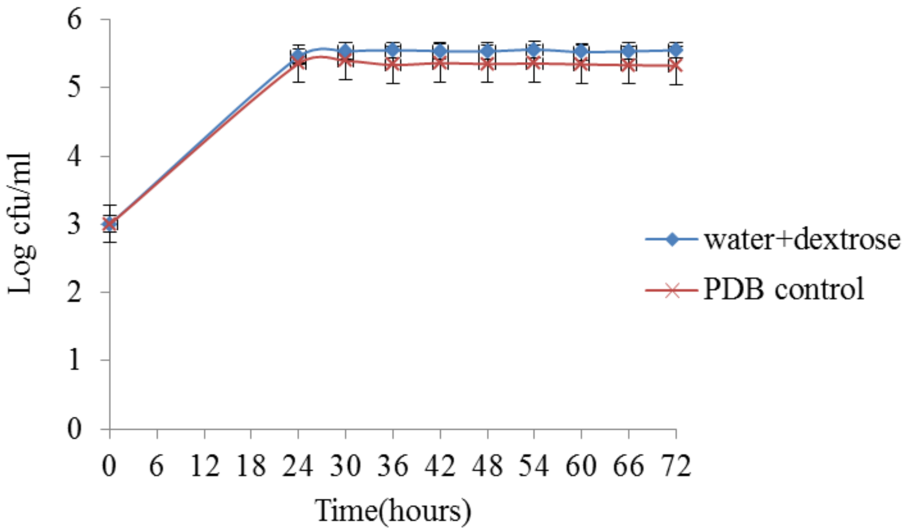


Figure 4.15 Comparison of growth models fitted to variable count data of mold grown at 30^{o} C between PDB and alternative media

4.3.1.2 Effect of nitrogen source on yeast/mold growth

Experimental Description

To study alternative media, three other nitrogen sources were tested to supplement PDB recipe (Figure 4.16). Growth kinetics of yeast and mold were monitored to investigate the effect of additional nitrogen supplement. The cultivation temperature was controlled at 30 ^{o}C. The total colony forming units were detected in the full well surface.

(a) Chicken (b) Mushroom (c) Yeast extract

Figure 4.16 Alternative media as nitrogen sources agent

Results and Discussion

Nitrogen constitutes primary organic sources for many organisms, including bacteria and fungi (Gerin et al., 2010). The overall impact of nitrogen source on yeast and mold growth by monitoring the viable colony count was evaluated. Table 4.11 showed the final viable colony count as the result of growing yeast and mold with different nitrogen supplements (e.g., chicken soup, mushroom soup and yeast extract treatments). The final colony count returned statistically the same number of final colony reading among different treatment comparable to the PDB control.

Table 4.11 Final colony enumeration of yeast and mold culturing by nitrogen-supplement PDB and control PDB

No. of yeast/mold count (log CFU/ml)		
Treatment	Yeast	Mold
PDB + chicken soup	5.31^{a} ±0.10	5.32^{a} ±0.13
PDB + Mushroom soup	5.49^{a} ±0.13	5.30^{a} ±0.05
PDB + yeast extract	5.49^{a} ±0.08	5.50^{a} ±0.10
PDB control	5.34^{a} ±0.10	5.49^{a} ±0.13

[a] values in a column with different superscripts are significantly difference at P<0.05.

The broth was formulated using three different nitrogen sources (chicken, mushroom and yeast extract). The investigation of viable yeast count over 48 hours was conducted to determine the growth profiles of different nitrogen treatments (Figure 4.17). Initially, the yeast density was started at approximately 2 log CFU/ml in all treatments. Yeast cells multiplied over the first 24 hours and reached the same plateau at 5 log CFU/ml. None of the treatments seemed to give different growth profiles comparing to the PDB control. This result showed the use of nitrogen supplements was not able to enhance or alter yeast growth kinetics. Nitrogen was not essential to PDB composition.

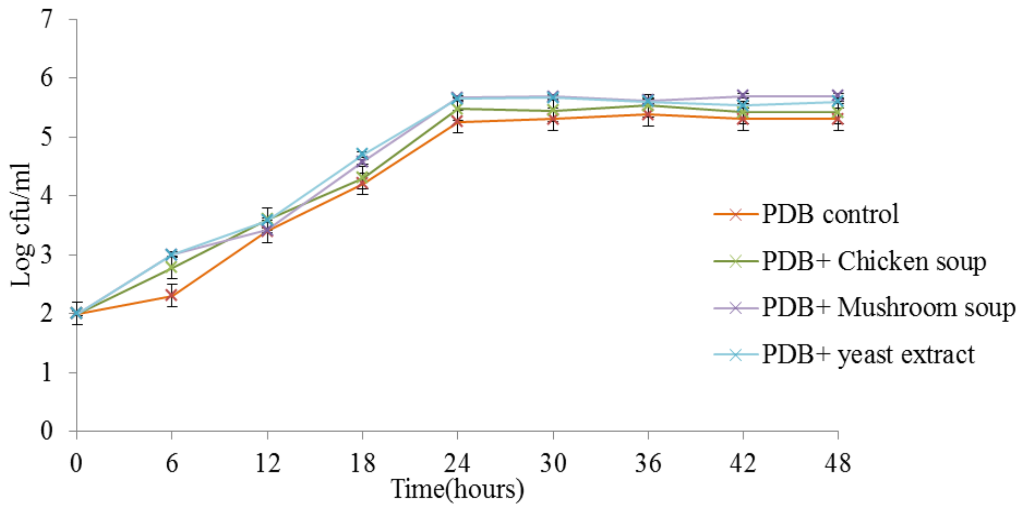


Figure 4.17 Comparison of growth models fitted to variable count data of yeast grown at 30° C between PDB and alternative media

In Figure 4.18, the experiment was setup using initial mold density at approximately 3 log CFU/ml. As seen earlier in the yeast experiment, the addition of nitrogen supplement was not able to improve mold growth in liquid PDB.

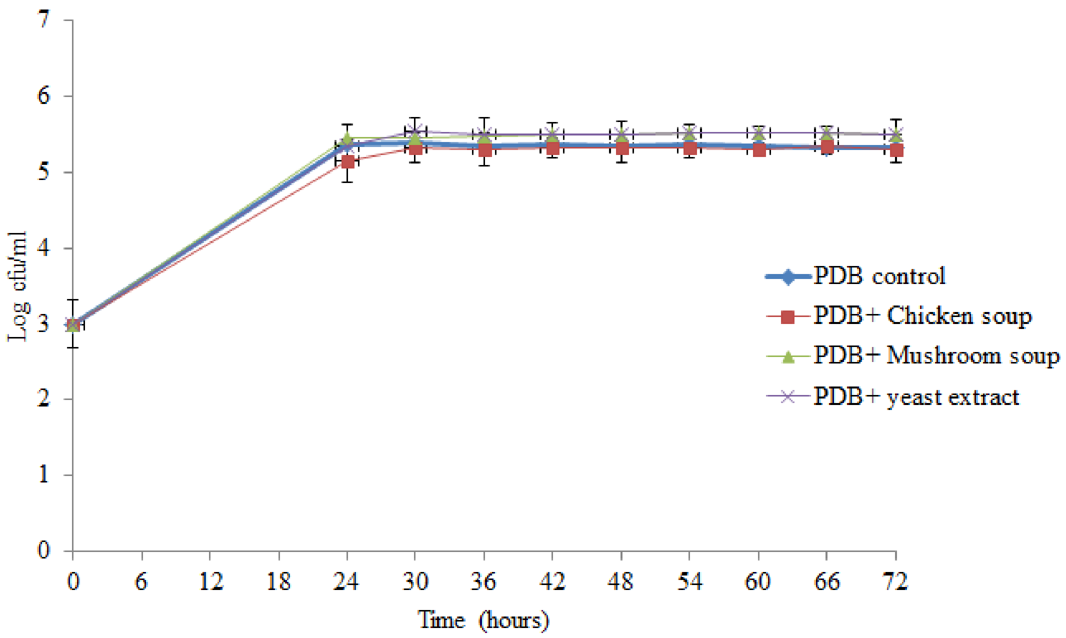


Figure 4.18 Comparison of growth models fitted to variable count data of mold grown at 30° C between PDB and alternative media

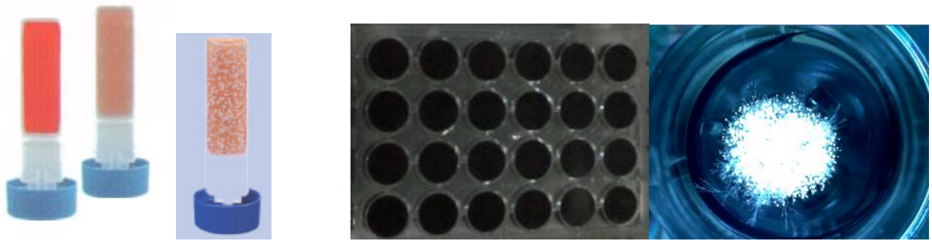
4.3.2 Implementation of yeast and mold enumeration on industrial plasticine and dough clay samples

Experimental Description

The micro-scale technique (Figure 4.19b) was used to perform yeast and mold count compared with traditional plating technique which are test kit (Figure 4.19a). The two strategies were examined and implemented to real-world samples, including plasticine and dough clay products. The 10 µl of each dilution were spread on micro-well agar for MIC, and dip the test kit in the sample for 5-10 seconds (wet the test kit under a running stream the liquid or spay the liquid on the test kit) and incubated at 30°C for three days (Easicult® Combi) and 24 h (MIC technique). The colony forming units were used to compare between methods.

Results and Discussion

Having the test kit as a designated standard method for most small- and medium-size factories in Thailand, the log CFU/ml results from the MIC method was evaluated as shown in Table 4.12 and 4.13. For most colony reading, the growth of colonies that favored oxidative pathways (the MIC technique) returned higher test kit readings (APHA, 2001; Buck and Cleverdon, 1960; Hoben and Somasegaran, 1982). However, most of these readings were insignificant no different in term of statistics since the deviations of the readings were fairly significant for all samples. The coherence of the yeast and mold reading suggested that these protocols were able to substitute each other for many industrial applications.



(a) (b)

Figure 4.19 The equipment of each technique was used to perform yeast and mold count (a) Easicult® Combi test kit (B) MIC technique

Table 4.12 Comparison of test kit and MIC for the enumeration of yeast and mold when perform on plasticine sample

Plasticine samples No.	No. of yeast/mold count (CFU/ml)	
	Test kit	MIC
1-5	$<10^2$	$<10^2$
6	10^3	$3.91 \pm 0.03 x 10^3$
7	10^3	$3.69 \pm 0.04 x 10^3$
8	10^3	$3.48 \pm 0.04 x 10^3$
9	10^3	$3.78 \pm 0.03 x 10^3$
10	10^3	$4.93 \pm 0.06 x 10^3$

Table 4.13 Comparison of test kit and MIC for the enumeration of yeast and mold when perform on dough clay sample

Dough clay samples No.	No. of yeast/mold count (CFU/ml)	
	Test kit	MIC
1-5	$<10^2$	$<10^2$
6	10^3	$5.12 \pm 0.16 x 10^3$
7	10^3	$4.21 \pm 0.11 x 10^3$
8	10^3	$2.51 \pm 0.16 x 10^3$
9	10^3	$3.22 \pm 0.11 x 10^3$
10	10^3	$2.30 \pm 0.13 x 10^3$

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This research demonstrated the development of micro-scale cultivation and digital microscopy-assisted technique to perform total plate count (TPC) yeast and mold detection. To develop the micro inoculation culture (MIC), extensive literature survey was performed to compare the advantages and disadvantages of the proposed method to the existing protocols (e.g., pour plate and spread plate techniques) as well as the commercialized rapid method that is the Petrifilms^{TM}. The engineering and microbiological aspects of these methodologies were critically analyzed to reduce analytical cost per sample and simplify the procedures and related equipment. The goal was to establish a simple-to-use technique and low capital investment and testing supplies. An application of visual aid can further lessen the detection time owing to higher magnification power using a series of magnifying lenses. The prototype digital microscopy was able to shorten the TPC colony detection time to approximately 6 - 8 h. In addition, the colony images were automatically converted to a digital format for future references and further analysis using digital image analytical tools. Such technique allows the evaluation of colony size and color.

A novel application of digital image analysis helped magnify the colony image and expedite rapid enumeration and identification. The proposed protocol was streamlined by improving protocol to detect industrial hygienic microbial contaminants. The standard calibration was also performed to validate the micro-inoculation technique (MIC) against the spread and pour plate techniques. This technology was successfully implemented to determine the total plate counts (TPC), yeast and mold of various types of factory samples, including finished frozen food products, production line swabs, plasticine and dough clay samples. For industrial samples, the MIC resulted in equivalent numbers of aerobic plate counts to the conventional techniques within 12-15 h as opposed to 24-48 h from the spread and pour plate techniques.

The size and characteristic of TPC colonies were observed using the prototype system of image analysis. The systematic improvement of colony detectability was demonstrated. This digital microscope enabled fast colony enumeration, which was linearly correlated with the reduction of colony incubations from generally 24 h to only 8 h. Agar color (i.e., green, red and yellow dye) of the media was used to enhance the contrast of the colony and the background. The contrast represents by delta E in the RGB system, which is the root-mean-square error of R, G and B indices. The red background provided the best color contrast over 60% larger delta E value compare to the traditional agar color.

The study of *E. coli* colony growth on CCA was facilitated by the use of a prototype digital imagery system. The highest colony expansion on CCA was obtained at a significantly higher incubation temperature setting (40^{o}C) than typical setting (35^{o}C) recommended by most microbiological standards and handbooks. The development of blue color attribute also followed the kinetics of colony area growth. The use of medium magnification digital microscopy allowed the detection of both colony and blue chroma within 8 hours suggesting the detection time of *E. coli* colony can be substantially reduced from overnight with human visual detection to 8 hours by microscopy-assisted inspection. The logistic model was very useful to extract important kinetic parameters of *E.coli* colony expansion. And the effect of CCA concentrations on *E. coli* color the size and purplish *E. coli* colonies was gradually changed as a result of the CCA dilution. At lower dilutions, the images of the *E. coli* colonies were very faint affecting colony numbers and the distinction of colony itself. For colony count purposes, the CCA preparation according to manufacturer's recommendation was able to reduce to 60-70% without compromising the colony detectivity. The tracking of the RGB color attributes as a function of culturing time indicated that the blue color attributes developed from beta-glucuronidase activity only differed when the CCA concentration went below 40%. The uses of the developed digital microscope were able to extent the dilution further and minimize the concentration of the CCA medium.

The optimization of the kinetics and growth characteristic of yeast and mold (i.e., *S. cereviseae* and *A. niger*) and the nutrient requirements (e.g., carbon and nitrogen prerequisites) were extensively scrutinized using Tukey test to differentiate the statistical differences. Mold growth kinetic and mathematical simulation showed no significant different when carbon and nitrogen sources were altered. Any nutrient broths from standard recipes were equivalently effective to resuscitate and encompassed sufficient nutrients for mold growth. The yeast growth, the dextrose solution returned fast growth in liquid media. Therefore, to grow yeast and mold in liquid culture, the cost of broth media can be drastically reduced using local carbon and nitrogen alternatives.

5.2 Recommendations

The results from this study showed an opportunity TPC yeast and mold detection by using the micro inoculation culture (MIC). However, the optimal temperature incubation and substrate dilution of CCA of this proposed detection procedure suggested from this study were performed on pure culture. The actual wild types or native strains may require longer incubation. The results from this study were only guideline for detection TPC, yeast and mold in food, plasticine, and dough clay samples. This research only included *Escherichia coli*, *Saccharomyces cerevisiae* and *Aspergillus niger*. More relevant strains of pathogenic and non-pathogenic need to be included in future studies. The implementation in real industrial application was required to gather more growth data and adjust the analytical procedure for practicality.

In term of mathematical method, though sigmoid model is suitable for detect maximum growth rate in this experiment but it cannot compare the result between sigmoid and others model to make more precise and accurate for predict the maximum growth rate next.

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APPENDIX A

Experimental Data

Enhancement of plate count agar technique

Table A.1 Comparison of the total cell count of *E. coli* at different background color of the medium using plate count agar (PCA)

treatment	*E. coli* count (log CFU/ml)					
	Rep1	Rep2	Rep3	Rep4	Ave	SD
Control	9.51	9.65	9.76	8.48	9.35	0.59
Green dye	9.65	9.87	9.03	9.26	9.45	0.38
Red dye	9.12	9.66	9.76	9.39	9.48	0.29
Yellow dye	9.49	9.55	9.13	9.39	9.39	0.19

Implementation of TPC enumeration on industrial samples

Table A.2 TPC enumeration in food samples using various techniques (pour plate, spread plate and MIC techniques)

Sample type	Sample No.	Total plate count (log CFU/ ml)											
		Pour plate				Spread plate				MIC			
		Rep1	Rep2	Ave	SD	Rep1	Rep2	Ave	SD	Rep1	Rep2	Ave	SD
Somtum	1-5	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	6-9	TNTC	TNTC	TNTC	-	TNTC	TNTC	TNTC	-	TNTC	TNTC	TNTC	-
	10	2.38	2.41	2.39	0.02	2.41	2.43	2.42	0.01	2.49	2.47	2.48	0.01
	11	2.78	2.72	2.75	0.04	2.88	2.81	2.85	0.05	2.78	2.65	2.72	0.09
	12	3.08	3.04	3.06	0.02	3.20	3.10	3.15	0.07	3.20	3.03	3.12	0.12
	13	2.81	2.76	2.79	0.04	2.88	2.82	2.85	0.04	2.90	2.85	2.88	0.03
	14	2.44	2.50	2.47	0.04	2.65	2.45	2.55	0.14	2.65	2.49	2.57	0.11
	15	2.88	2.77	2.83	0.08	2.90	2.87	2.89	0.02	2.90	2.87	2.89	0.02
Rice	1-11	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	12	2.89	2.76	2.83	0.09	2.98	2.78	2.88	0.14	2.98	2.80	2.89	0.13
	13	2.65	2.59	2.62	0.04	2.72	2.65	2.69	0.05	2.72	2.62	2.67	0.07
	14	2.45	2.65	2.55	0.14	2.60	2.58	2.59	0.01	2.60	2.43	2.52	0.12
	15	2.68	2.62	2.65	0.04	2.61	2.65	2.63	0.03	2.70	2.68	2.69	0.01
Topping	1	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	2	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	3	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	4	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
	5	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-

Table A.3 TPC enumeration in production lines using various techniques (pour plate, spread plate and MIC techniques)

Sample	Total Plate Count (log CFU/ ml)											
	Pour plate				Spread plate				MIC			
	Rep1	Rep2	Ave	SD	Rep1	Rep2	Ave	SD	Rep1	Rep2	Ave	SD
Plastic curtain	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
Worker glove	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
Worker hands	ND	ND	ND	-	ND	ND	ND	-	ND	ND	ND	-
Tray before freezing	TNTC	TNTC	TNTC	-	4.25	4.39	4.32	0.10	4.31	4.33	4.32	0.01
Stainless steel tray	2.44	2.64	2.54	0.14	2.85	3.05	2.95	0.14	3.35	2.87	3.11	0.34

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Effect of temperature on TPC growth

Table A.4 Comparison of the total cell count of *E. coli* at different incubation temperatures using Chromocult$^{®}$ Coliform Agar

Incubation		*E. coli* count (log cfu/ ml)				
temperature	Rep 1	Rep 2	Rep 3	Rep 4	Ave	SD
30^{o}C	8.39	8.39	8.30	8.30	8.35	0.06
35^{o}C	8.30	8.39	8.30	8.39	8.35	0.06
37^{o}C	8.48	8.40	8.40	8.48	8.44	0.05
40^{o}C	8.48	8.48	8.54	8.48	8.50	0.03
45^{o}C	0.00	0.00	0.00	0.00	0.00	0.00

Kinetics of *E.coli* colony expansion

Table A.5 Growth kinetics of *E.coli* on Chromocult® Coliform Agar and relative coefficient of logistic model at different incubation temperatures (μ_{max})

Incubation temperatuers	Growth kinetic parameters					
	Rep1	Rep2	Rep3	Rep4	Ave	SD
30^{o}C	0.162	0.164	0.165	0.162	0.163	0.002
35^{o}C	0.165	0.162	0.163	0.165	0.163	0.002
37^{o}C	0.175	0.174	0.172	0.173	0.174	0.002
40^{o}C	0.200	0.202	0.203	0.198	0.200	0.002
45^{o}C	0.00	0.00	0.00	0.00	0.00	0.00

Table A.6 Growth kinetics of *E.coli* on Chromocult® Coliform Agar and relative coefficient of logistic model at different incubation temperatures (X_{max})

Incubation	Growth kinetic parameters					
temperatuers	Rep1	Rep2	Rep3	Rep4	Ave	SD
30°C	17313	17205	17318	17108	17236	100
35°C	20016	20800	20001	20206	20256	375
37°C	26304	26958	25914	26121	26324	456
40°C	32439	31845	32966	32515	32441	461
45°C	0.00	0.00	0.00	0.00	0.00	0.00

Kinetics of *E.coli* color development

Table A.7 Comparison of key kinetic parameters in term of color development (delta blue) (μ_{max})

Incubation	Growth kinetic parameters					
temperatuers	Rep1	Rep2	Rep3	Rep4	Ave	SD
30°C	0.878	0.895	0.832	0.865	0.868	0.027
35°C	0.991	1.012	1.013	0.993	1.002	0.019
37°C	0.883	0.799	0.821	0.930	0.860	0.059
40°C	1.129	1.125	1.122	1.098	1.119	0.014
45°C	0.00	0.00	0.00	0.00	0.00	0.00

Table A.8 Comparison of key kinetic parameters in term of color development (delta blue) (X_{max})

Incubation	Growth kinetic parameters					
temperatuers	Rep1	Rep2	Rep3	Rep4	Ave	SD
30^{o}C	9087	9018	9199	9047	9087.750	75.378
35^{o}C	9155	9207	9137	9168	9166.750	29.691
37^{o}C	9916	9877	9938	9931	9915.500	27.258
40^{o}C	10227	10342	10109	10229	10226.750	95.136
45^{o}C	0.00	0.00	0.00	0.00	0.00	0.00

Effect of substrate dilution and nutrient limitation on *E.coli* growth

Table A.9 Colony count on culture plates using different concentrations of CCA comparing to the original concentration

CCA dilution	*E. coli* count (log CFU/ ml)					
	Rep 1	Rep 2	Rep 3	Rep 4	Ave	SD
10%	9.12	9.13	9.19	9.05	9.11	0.04
20%	9.21	9.28	9.21	9.19	9.22	0.04
30%	9.26	9..35	9.23	9.20	9.26	0.06
40%	9.25	9.28	9.29	9.32	9.29	0.03
50%	9.24	9.29	9.27	9.32	9.28	0.03
60%	9.28	9.30	9.29	9.31	9.30	0.01
70%	9.28	9.29	9.32	9.26	9.29	0.03
80%	9.29	9.32	9.31	9.32	9.31	0.01
90%	9.29	9.28	9.31	9.32	9.30	0.02
Original conc.	9.31	9.30	9.28	9.31	9.30	0.01

Optimization of liquid substrate for yeast/mold

Table A.10 Comparison of alternative media for the enumeration of yeast/mold when perform on pure culture

Treatments	No. of yeast/mold count (log CFU/ml)									
	Yeast					Mold				
	Rep1	Rep2	Rep3	Ave	SD	Rep1	Rep2	Rep3	Ave	SD
Long grain rice flour	5.23	5.35	5.32	5.30	0.06	5.27	5.21	5.08	5.18	0.09
Corn flour	5.15	5.11	5.28	5.18	0.08	5.15	5.36	5.27	5.26	0.10
Cassava flour	5.23	5.21	5.20	5.21	0.01	5.07	4.81	4.97	4.95	0.13
Wheat flour	5.53	5.61	5.40	5.51	0.10	5.15	5.22	5.24	5.20	0.04
Glutinous rice flour	5.05	5.30	5.27	5.20	0.13	5.35	5.02	5.30	5.22	0.17
Long grain rice	5.09	5.20	5.27	5.19	0.09	5.25	5.32	5.22	5.26	0.05
Glutinous rice	5.09	5.25	5.26	5.20	0.09	5.20	5.04	5.00	5.08	0.10
PDB control	5.59	5.45	5.56	5.53	0.07	5.49	5.22	5.32	5.34	0.13

Table A.11 Viable cell counts of yeast and mold comparing pure dextrose to PDB control media using pure cultures

Treatments	No. of yeast/mold count (log CFU/ml)									
	Yeast					Mold				
	Rep1	Rep2	Rep3	Ave	SD	Rep1	Rep2	Rep3	Ave	SD
Water + dextrose	5.80	5.99	5.48	5.76	0.26	5.67	5.47	5.52	5.55	0.10
PDB control	5.55	5.12	5.38	5.30	0.22	5.20	5.30	5.47	5.32	0.13

Table A.12 Final colony enumeration of yeast and mold culturing by nitrogen-supplement PDB and control PDB

Treatments	No. of yeast/mold count (log CFU/ml)									
	Yeast					Mold				
	Rep1	Rep2	Rep3	Ave	SD	Rep1	Rep2	Rep3	Ave	SD
PDB + chicken soup	5.41	5.21	5.33	5.31	0.10	5.31	5.20	5.46	5.32	5.13
PDB + Mushroom soup	5.45	5.64	5.39	5.49	0.13	5.25	5.32	5.35	5.30	5.05
PDB + yeast extract	5.49	5.41	5.58	5.49	0.08	5.41	5.62	5.49	5.50	5.10
PDB control	5.31	5.25	5.46	5.34	0.10	5.45	5.64	5.39	5.49	5.13

Implementation of yeast and mold enumeration on industrial plasticine and dough clay samples

Table A.13 Comparison of test kit and MIC for the enumeration of yeast and mold when perform on plasticine sample

Sample No.	No. of yeast/mold count (CFU/ml)				
	Plasticine				
	Rep1	Rep2	Rep3	Ave	SD
1-5	ND	ND	ND	ND	-
6	3.91	3.95	3.89	3.91	0.03
7	3.75	3.66	3.68	3.69	0.04
8	3.47	3.53	3.45	3.48	0.04
9	3.79	3.74	3.81	3.78	0.03
10	4.93	4.99	4.87	4.93	0.06

Table A.14 Comparison of test kit and MIC for the enumeration of yeast and mold when perform on dough clay sample

Sample No.	No. of yeast/mold count (CFU/ml)				
	Dough clay				
	Rep1	Rep2	Rep3	Ave	SD
1-5	ND	ND	ND	ND	-
6	5.05	5.31	5.01	5.12	0.16
7	4.33	4.11	4.20	4.21	0.11
8	2.71	2.43	2.41	2.51	0.16
9	3.34	3.22	3.12	3.22	0.11
10	2.40	2.15	2.37	2.30	0.13

CURRICULUM VITAE

NAME	Mrs. Orana Rattanabumrung
DATE OF BIRTH	18 June 1987
EDUCATIONAL RECORD	
HIGH SCHOOL	Kanlayanee Si Thammarat School, 2005
BACHELOR’S DEGREE	Bachelor of Engineering (Food Engineering) King Mongkut’s Institute of Technology Ladkrabang, 2009
MASTER’S DEGREE	Master of Engineering (Food Engineering) King Mongkut’s University of Technology Thonburi, 2012
SCHOLARSHIP/ RESEARCH GRANT	Food Engineering Scholarship, 2010-2011 The Thailand Research Fund-Master Research Grants (TRF-MAG Window I) 2554
PUBLICATIONS	Rattanabumrung O., Sangadkit W., Supanivatin, P. and Thipayarat A., 2012, Visual improvement of colony detectability for high-throughput total plate count protocol, **Proceedings of The 23rd Annual Meeting of the Thai Society of Biotechnology Systems Biotechnology,** pp. 31-32.

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