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

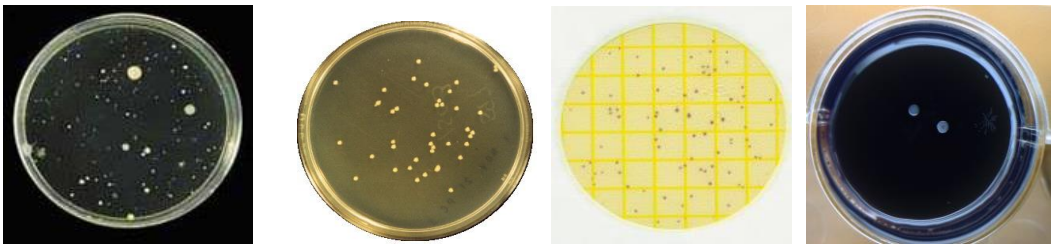
Experimental Description

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^{TM} 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^{TM} 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^{TM} 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^{TM}	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).

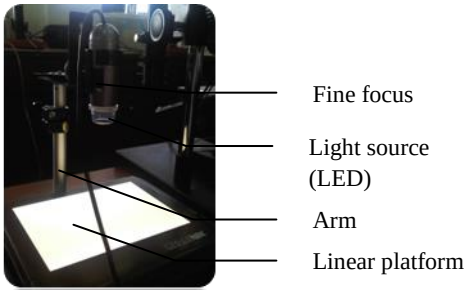


Figure 4.2 Photographs of the MIC equipment used to perform miniaturized TPC experiment

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.

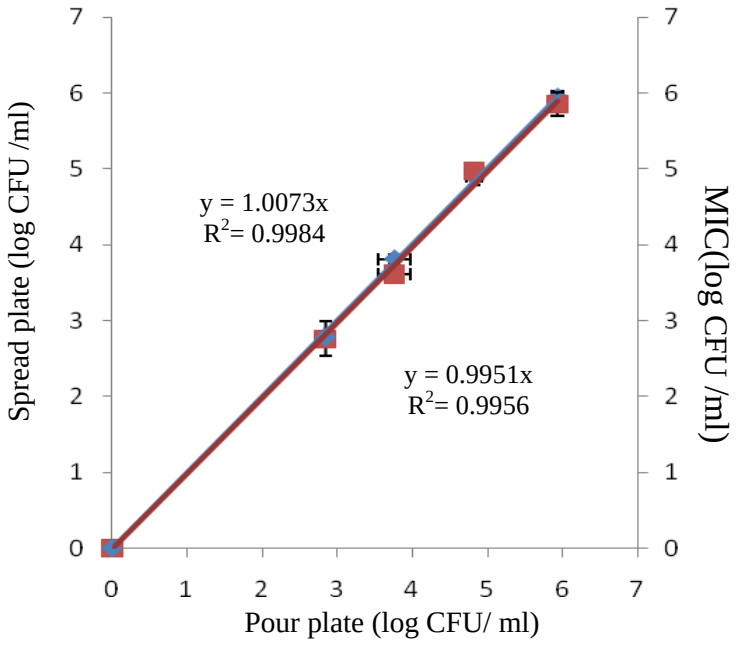
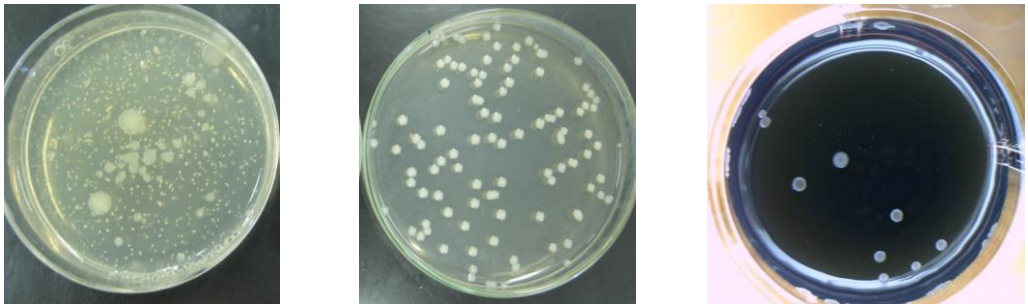


Figure 4.3 Correlation between estimates of TPC obtained by Pour- Spread plate (△) and Pour plate- MIC (X)

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

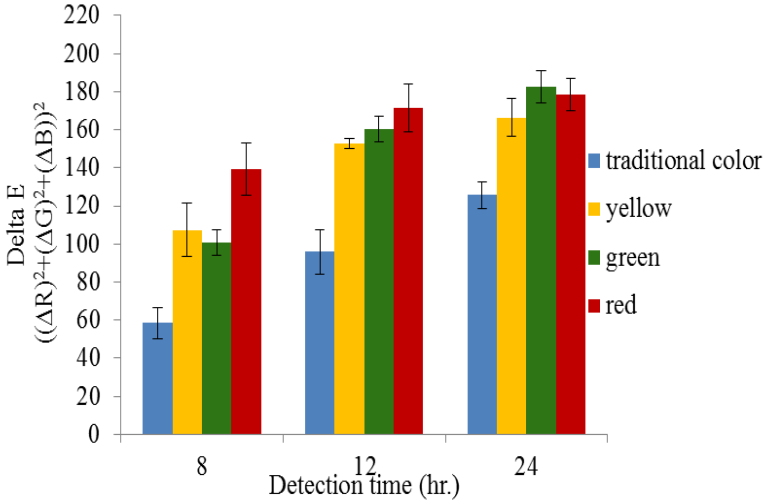
PCA mixture was prepared at 500 ml and mixed with 6.25 ml of different dye pigments (i.e., green, red and yellow colors). The PCA solution was autoclaved at 121^{o}C for 15 min and formed culture plates for *E. coli* cultivation. The incubation was maintained at 37^{o}C for 24 h. The size and characteristic 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.

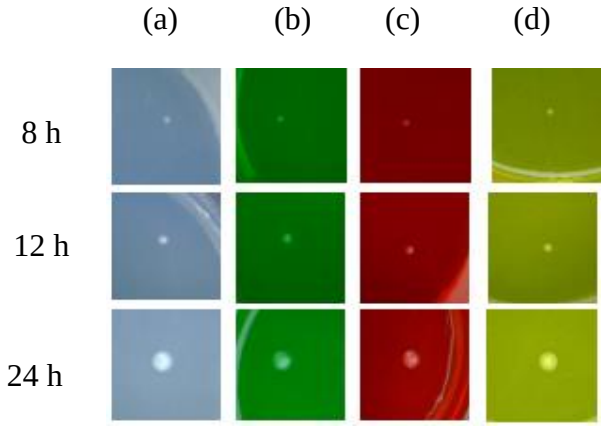


Figure 4.6 Photographs of colony formed on plate count agar using different background color of the medium (a) control; (b) green dye pigment; (c) red dye pigment and (d) yellow dye pigment

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]±0.02	2.42[b]±0.01	2.48[b]±0.01
	11	2.75[a]±0.04	2.85[a]±0.05	2.72[a]±0.09
	12	3.06[b]±0.02	3.15[b]±0.07	3.12[a]±0.12
	13	2.79[a]±0.04	2.85[a]±0.04	2.88[a]±0.03
	14	2.47[a]±0.04	2.55[b]±0.14	2.57[b]±0.11
	15	2.83[a]±0.08	2.89[a]±0.02	2.89[a]±0.02
Rice	1-11	ND[a]	ND[a]	ND[a]
	12	2.83[a]±0.09	2.88[a]±0.14	2.89[a]±0.13
	13	2.62[a]±0.04	2.69[a]±0.05	2.67[a]±0.07
	14	2.55[a]±0.14	2.59[a]±0.01	2.52[a]±0.12
	15	2.65[a]±0.04	2.63[a]±0.03	2.69[a]±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 45ºC) 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^{o}C), no *E. coli* cell survived on CCA. At the temperature between 30-40^{o}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^{o}C	35^{o}C	37^{o}C	40^{o}C	45^{o}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.

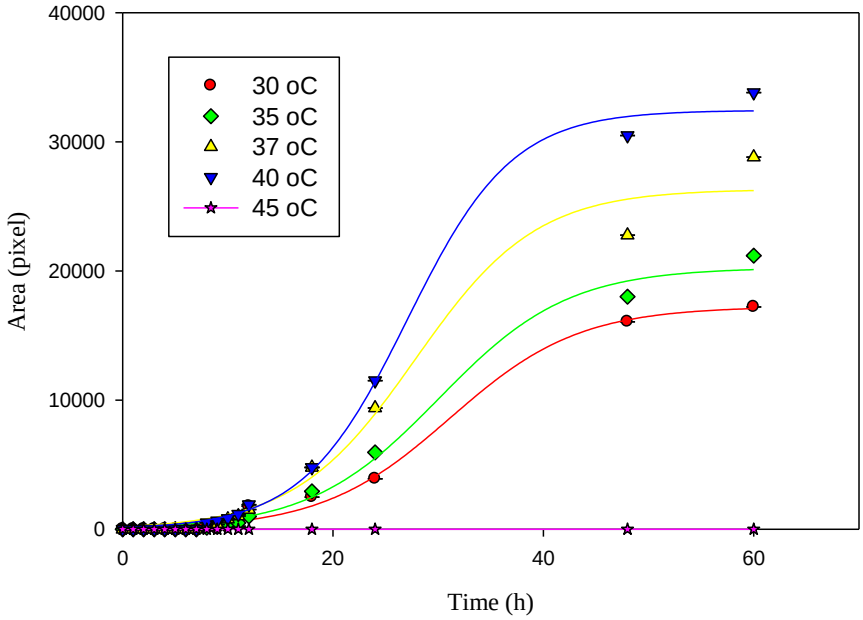


Figure 4.7 Profiles of colony growth in five different incubation conditions (red line: 30^{o}C; green line: 35^{o}C; yellow line: 37^{o}C; blue line: 40^{o}C and pink line: 45^{o}C)

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^{o}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^{o}C substantially diminished the intrinsic growth kinetics. At low incubation temperature (e.g., 30^{o}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.

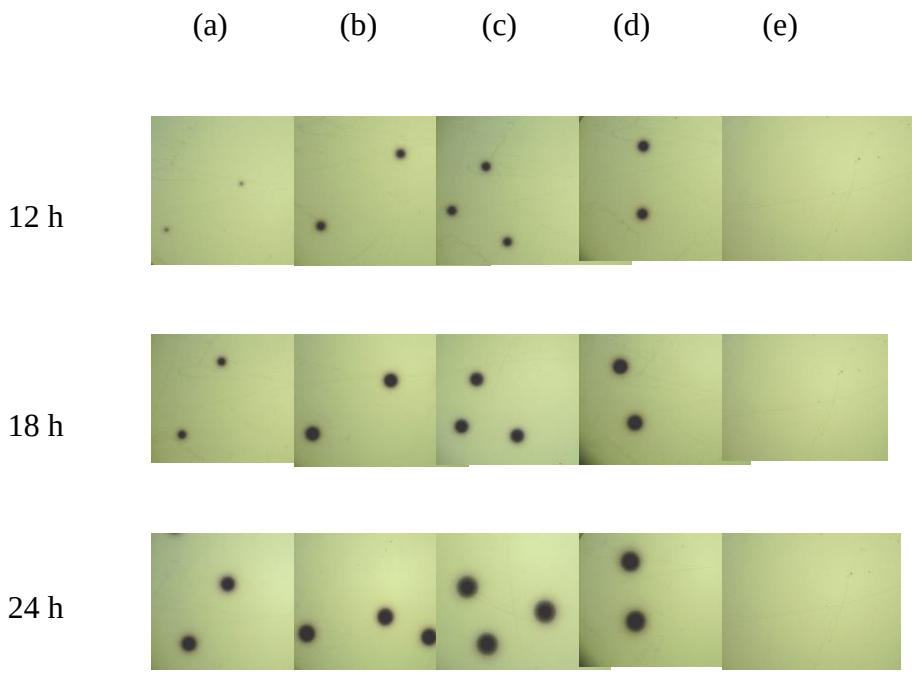


Figure 4.8 Photographs of colony formed on plate count agar using different incubation conditions (a) 30^{o}C; (b) 35^{o}C; (c) 37^{o}C; (d) 40^{o}C and (e) 45^{o}C

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^{o}C rather than 35-37^{o}C) to expedite blue chroma development. Both maximum specific B attribute development rate and final blue chroma were maximized at the 40^{o}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.

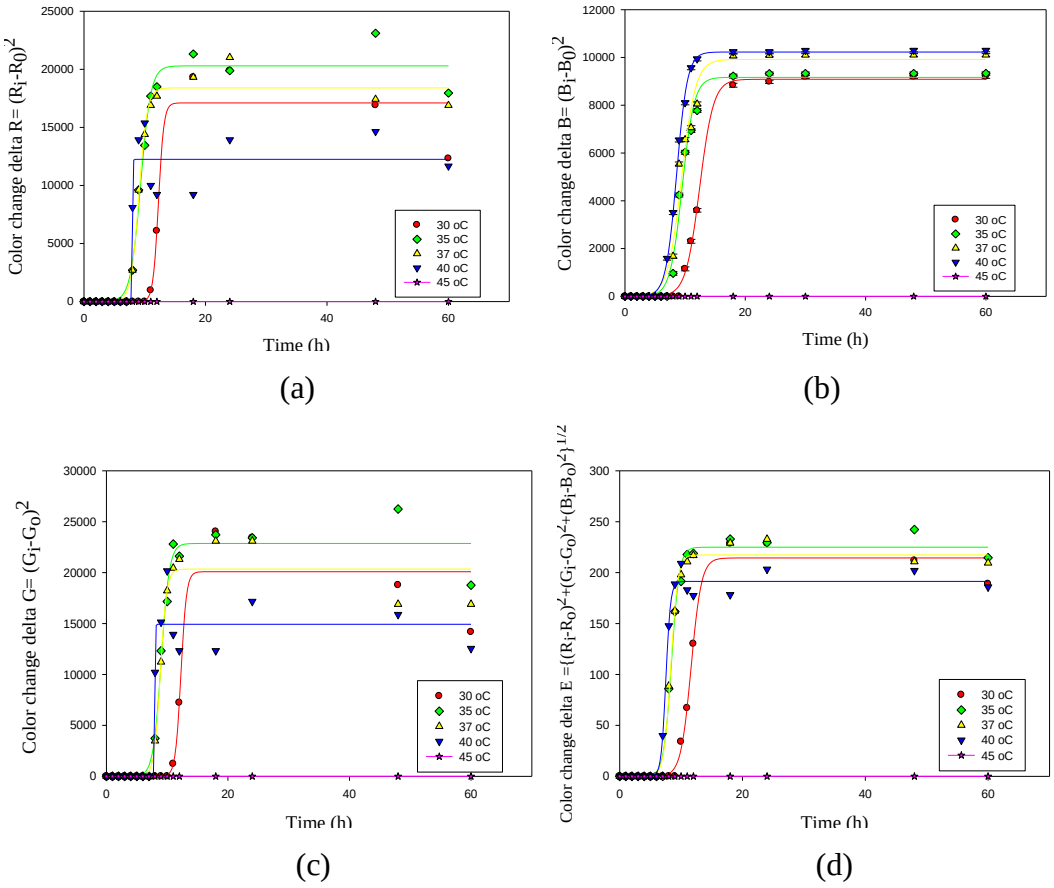


Figure 4.9 Profiles of color change using different incubation temperatures (a) delta R; (b) delta B; (c) delta G and (d) overall color change or delta E

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).

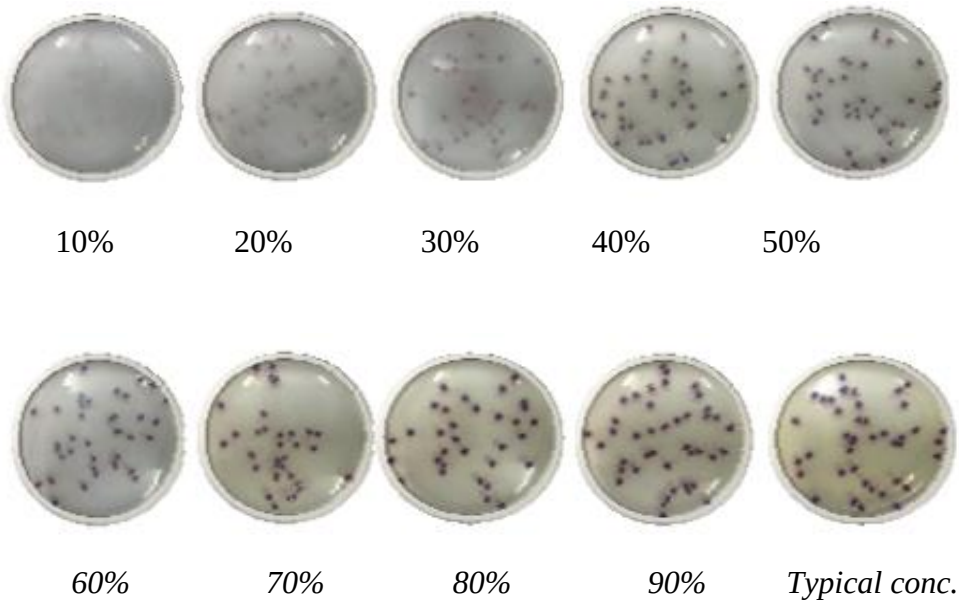


Figure 4.10 Digitized images of *E. coli* colony from pure culture after 12 h of incubation at 35±2°C

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.

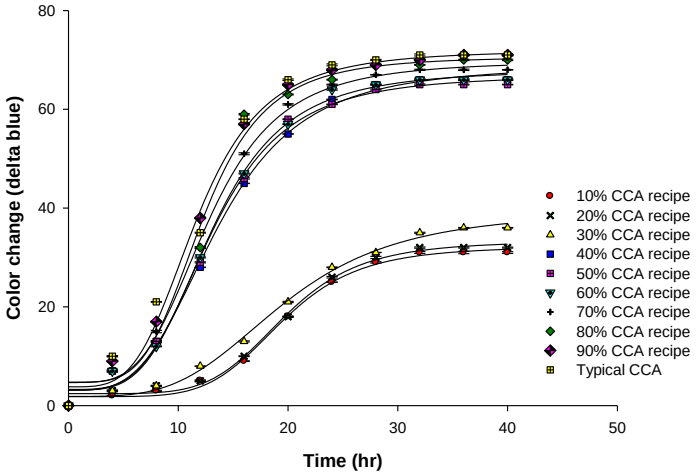


Figure 4.11 Profiles of color change (delta blue) of *E. coli* colonies as a result of varying CCA concentrations

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.

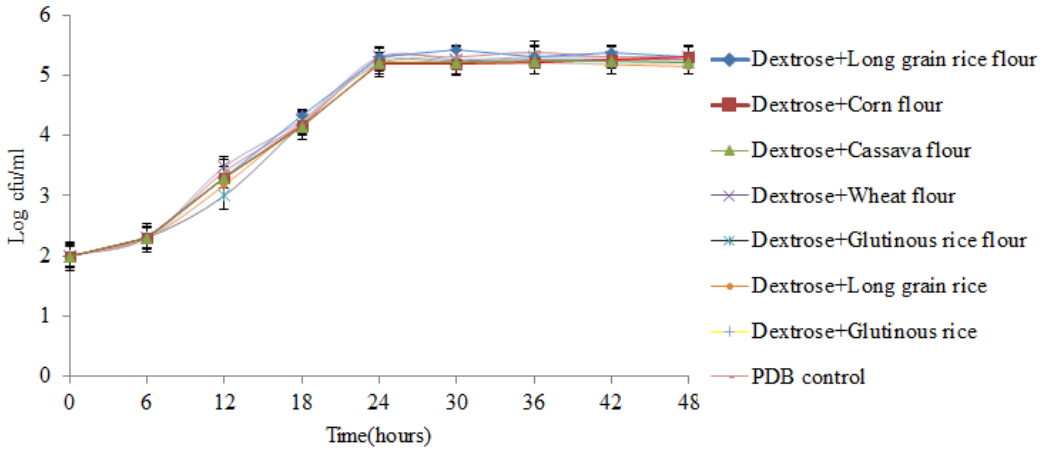


Figure 4.12 Comparison of growth models fitted to variable count data of yeast grown at 30° C between PDB and alternative media

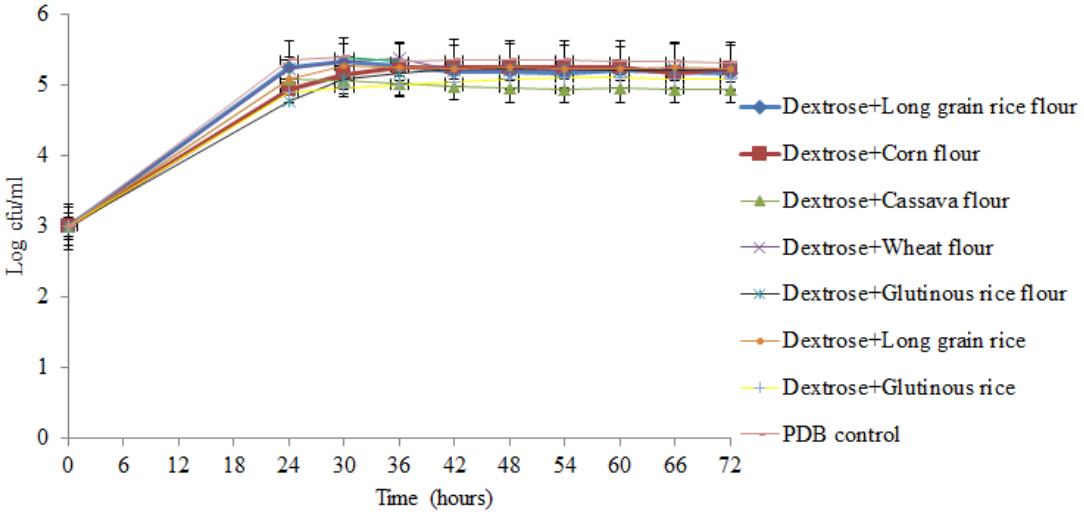


Figure 4.13 Comparison of growth models fitted to variable count data of mold grown at 30° C between PDB and alternative media

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.

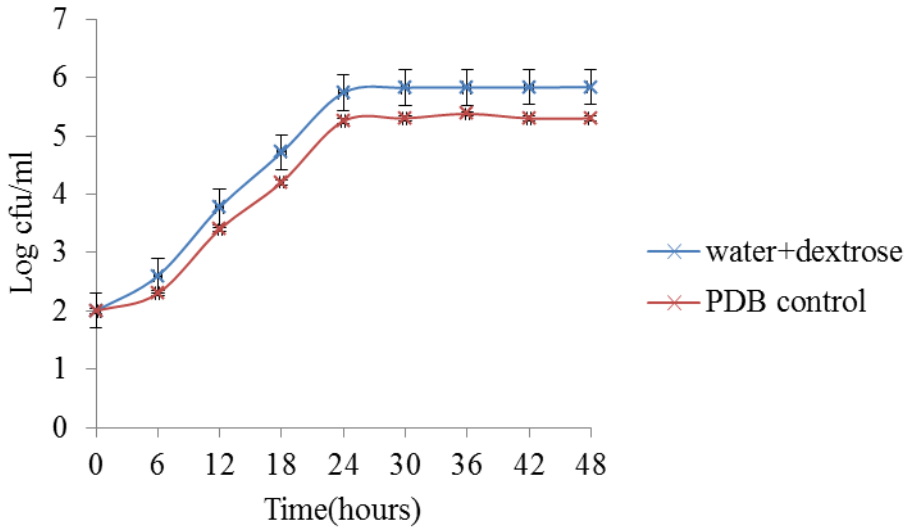


Figure 4.14 Comparison of growth models fitted to variable count data of yeast grown at 30^{o} C between PDB and alternative media

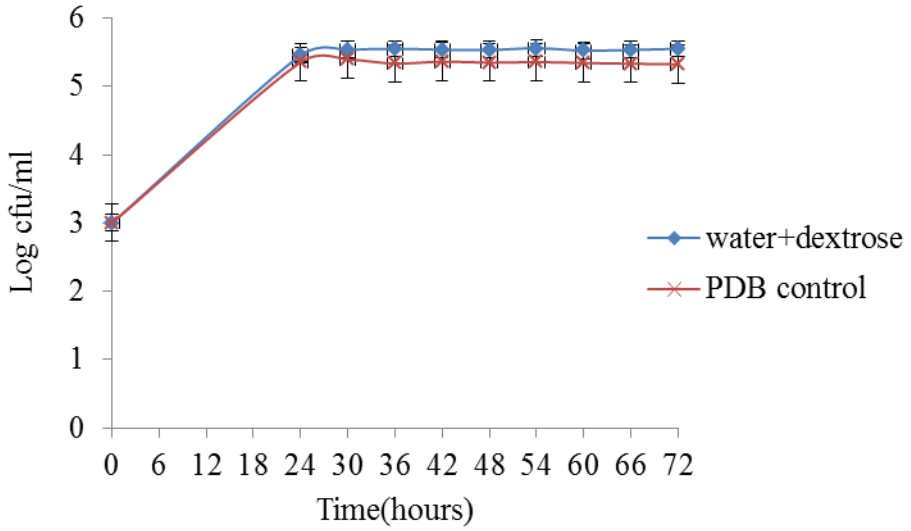


Figure 4.15 Comparison of growth models fitted to variable count data of mold grown at 30^{o} C between PDB and alternative media

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.

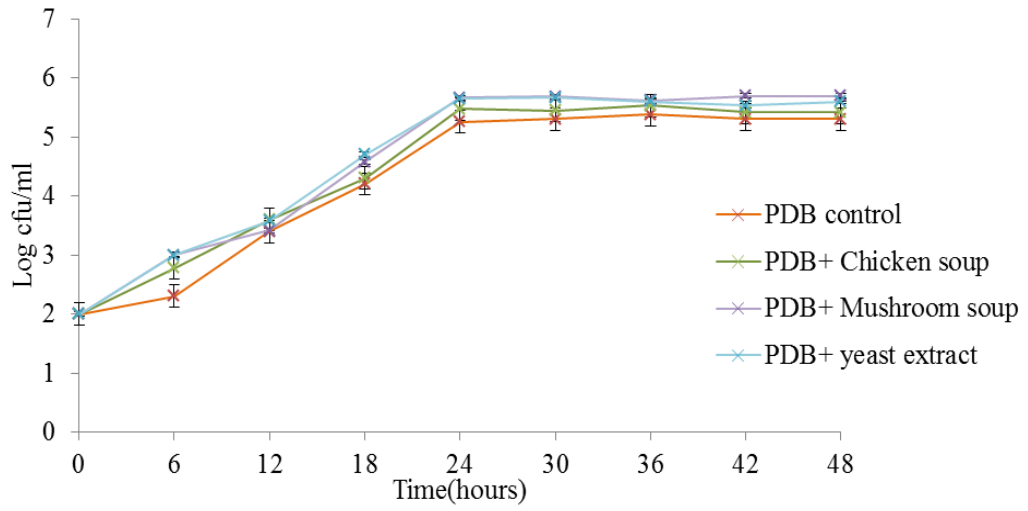


Figure 4.17 Comparison of growth models fitted to variable count data of yeast grown at 30° C between PDB and alternative media

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.

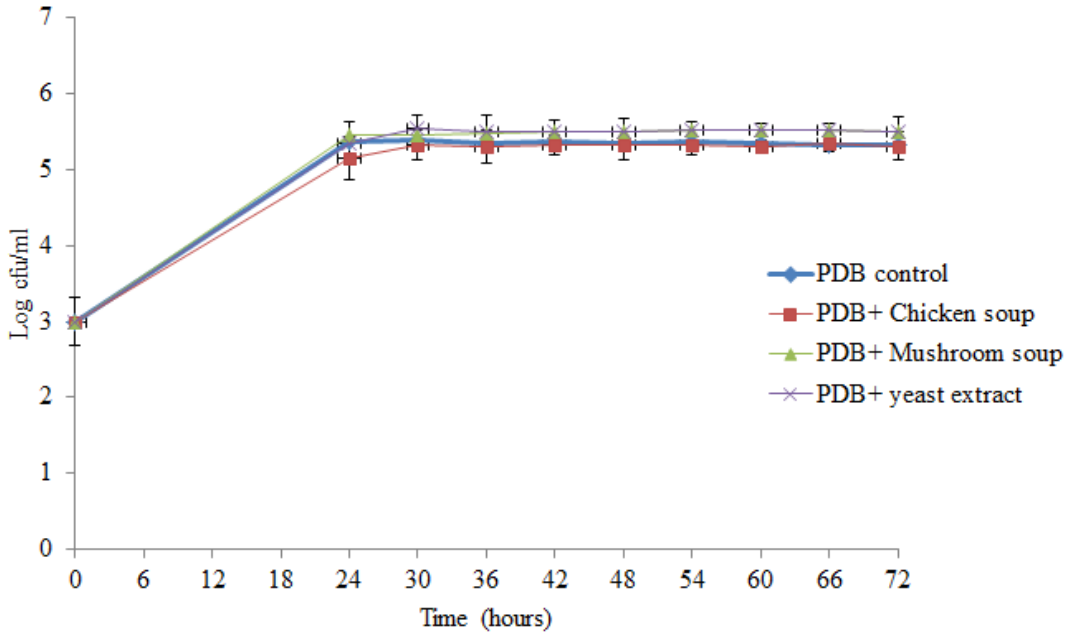


Figure 4.18 Comparison of growth models fitted to variable count data of mold grown at 30° C between PDB and alternative media

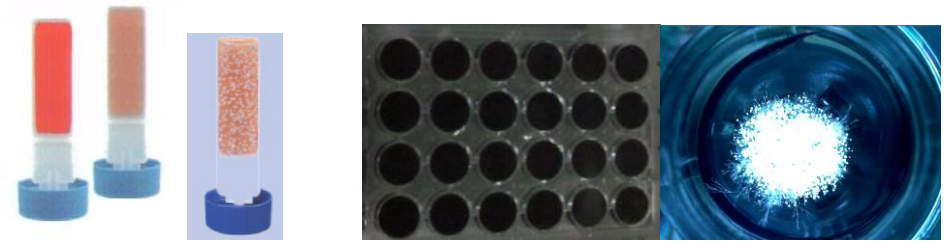
4.3.2 Implementation of yeast and mold enumeration on industrial plasticine and dough clay samples

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	$3.91\pm0.03x10^3$
7	10^3	$3.69\pm0.04x10^3$
8	10^3	$3.48\pm0.04x10^3$
9	10^3	$3.78\pm0.03x10^3$
10	10^3	$4.93\pm0.06x10^3$

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	$5.12\pm0.16x10^3$
7	10^3	$4.21\pm0.11x10^3$
8	10^3	$2.51\pm0.16x10^3$
9	10^3	$3.22\pm0.11x10^3$
10	10^3	$2.30\pm0.13x10^3$