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 (α=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^{o}C for 15 or 60 minutes by using autoclave and classical boiling at 95^{o}C for 60 minutes as well as mushroom extraction.

Vary Alternative Media Types

All alternative media were extracted by using autoclave at 121^{o}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.

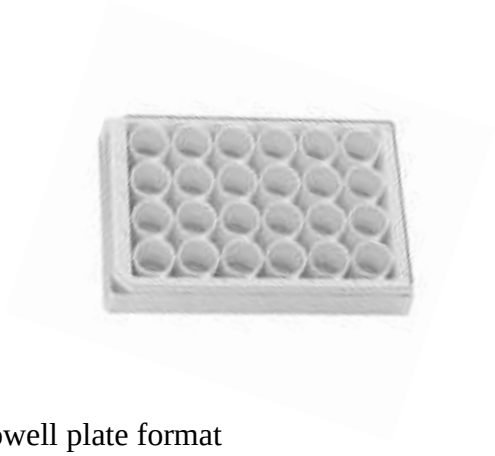

Figure 3.2 24-microwell plate format

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