Response Surface Methodology for Disinfection of *Raoultella terrigena* TISTR 1568 in Water with Chlorine

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Abstract. The aim of this research was to develop technique to disinfect bacteria in water with chlorine. Response surface methodology (RSM) was applied for investigating the experimental design for disinfection the *Klebsiella terrigena* (*Raoultella terrigena* TISTR 1568). There were 20 experiments involving the three investigated variables of chlorine concentration, pH and disinfection time that were studied on *R. terrigena* TISTR 1568 to optimize the condition for disinfecting the *R. terrigena* TISTR 1568 to 99.9% death. Design of experiment was performed by application of 5-level-3-factors central composite design in order to study the optimum condition for disinfecting the *R. terrigena* TISTR 1568 to 99.9% death. The investigated results showed that the optimum condition was 1.2 ppm of chlorine concentration, at pH 7 and 5 min of disinfection time.

1. Introduction
Chlorine disinfectants have been widely and effectively used to protect the safety of drinking water. Due to concern over suspected carcinogens formed by chlorine use, water utilities are increasingly switching from chlorine to chloramine for residual disinfection [1]. Disinfection by-products (DBPs) generated by the reaction between chlorine with organic matter are an important concern for water supply. Especially surface water is generally rich in nature organic matter. Chloramine is generally thought to produce a more stable residual than free chlorine and thus provides lasting protection against regrowth. Therefore, the objective of this study was to investigate the effect of chlorine concentration, pH and disinfection time on the amount of bacteria for optimizing condition of disinfection with chlorine that was used as first disinfectant before using the chloramine from chlorine with ammonia for lasting disinfectant.

*Raoultella terrigena* known as *Klebsiella terrigena*, was selected as bacteria for studying. It is a Gram-negative bacterial species of the genus *Klebsiella* and mainly reported as aquatic and soil organism but rarely from humans [2]. There were studies about this species. It has shown no connection with disease in humans despite expressing many of the virulence factors expressed by other *Klebsiella* species.
RSM (Response surface methodology) is a useful statistical technique which has been applied in research into complex variable process. The multiple regression and correlation analyses are used as tools to assess the effect of two or three independent factors on the dependent variables. In addition, the CCD (Central composite design) of RSM has been applied in the optimization of several biotechnological and chemical processes. The principle advantage of RSM is the reduced number of experimental runs required to generate sufficient information for a statistically acceptable result. RSM has been successfully applied in the study and optimization of parameters for water and wastewater treatment [3, 4, 5, 6]. In this work, the Klebsiella terrigena (R. terrigena TISTR 1568) was used as bacteria for disinfecting in water with chlorine to study the optimized condition by RSM to disinfect the R. terrigena TISTR 1568 to 99.9% death.

2. Materials and Methods

2.1 Materials

K. terrigena (R. terrigena TISTR 1568) was collected from TISTR Culture Collection Centre of Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research (TISTR) in Thailand. The R. terrigena TISTR 1568 was prepared at 0.85% sterilized sodium chloride (NaCl) for 1.5×10^8 CFU/ml R. terrigena TISTR 1568 concentration. The chemicals for this research are Sodium hypochlorite (NaOCl) 12.5% w/v, Ammonia (NH3), Buffered Peptone Water, Distilled Water, Sodium chloride (NaCl) 0.85%, Nutrient Agar (NA), etc. They are analytical grade. The reference standard of McFarland is 0.5 McFarland from bioMerieux, Inc, (USA).

McFarland Standard is a series of standards of different opacity, allowing the estimation of the density of bacterial suspensions. Its use is necessary for the standardization of microbiological methods. These standards are designated by the tube numbers of the scale initially described by McFarland. The density of the bacterial suspension is checked by comparison with a suspension of known opacity contained in an ampule of the same diameter. The composition of 0.5 McFarland Standard is 2.40×10^-5 mol/l BaSO4.

2.2 Apparatus and disinfection Procedures

The disinfections were conducted in 250 ml Erlenmeyer flask with 10 ml of 1.5×10^8 CFU/ml of R. terrigena TISTR 1568. It was compared with 0.5 McFarland Standard. A certain quantity of chlorine solution, a certain pH value adjusted with H2SO4 for acid and NH3 for alkaline were added to the flask. The flask was shaken with hand fast. After achieving the 1.5×10^8 CFU/ml of R. terrigena TISTR 1568, chlorine solution and a pH of solution were add to the flask, the disinfection was timed immediately. When the disinfection completed, took the 0.1 ml of solution to spread plate of Nutrient agar and 1 ml of solution to the 8 dram bottle with 9 ml of buffer peptone water. Diluting the solution to 10^{-1}-10^{-7} R. terrigena TISTR 1568 and took the 0.1 ml of solution to spread plate of nutrient agar. The plate culture of solution was incubated at 30°C, 24 hours after counting the colony of R. terrigena TISTR 1568 on the plate culture.

2.3 Experimental Design for disinfection of R. terrigena TISTR 1568 in water with chlorine

A five-level and three-factor CCD with 20 experiments was employed in this study. Chlorine concentration (C), pH (P) and disinfection time (T) were the independent variables to optimize the disinfection of R. terrigena TISTR 1568 in water with chlorine. The coded and uncoded levels of the independent variables, independent factor, levels and experimental design were shown in table 1. The central values (zero level) chosen for experimental design were 1.0 (ppm) of chlorine concentration, 7.00 of pH and 25 min of disinfection time.

Table 1. Independent variables and levels used for CCD for disinfection of R. terrigena TISTR 1568.

| Variables | Symbols | Code levelsa |
|-----------|---------|--------------|
|           | -1.68   | -1           |
|           | 0       | +1           |
| (Uncoded) | (-α)    | (+ α)        |

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Variables: C (Chlorine concentration), P (pH), T (Disinfection time).

a Code levels: Coded and uncoded levels.
Chlorine concentration & C & 0.16 & 0.5 & 1.0 & 1.5 & 1.84  \\

pH & P & 4.48 & 5.50 & 7.00 & 8.50 & 9.52  \\

Disinfection time (min) & T & 1.48 & 11 & 25 & 39 & 48.52  \\

Transformation of variable levels from code (X) to uncoded could be obtained as: $C = 1 + 0.5 \, X, P = 7 + 1.5 \, X$ and $T = 25 + 14 \, X$.

### 2.4 Statistical Analysis

The experimental data of the disinfection of *R. terrigena* TISTR 1568 in water with chlorine was performed by the RSM that following a second-order polynomial equation generated SPSS (Statistical package for social science) software. The suitable model was used to generate a response surface plot by STATISTICA software. The quadratic response surface model was shown in equation (1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j$$

(1)

Where $Y$ is the response (*R. terrigena* TISTR 1568 (log)), $\beta_0$ is constant, $X_i$ and $X_j$ are the independent variables, $\beta_i$ and $\beta_j$ are the linear term coefficients, $\beta_{ii}$ is quadratic term coefficients, and $\beta_{ij}$ is the interaction constant coefficients. SPSS package was used for regression ANOVA (analysis of variance) and RSM was performed using the SPSS software [7].

### 3. Results and Discussion

#### 3.1 Optimization of disinfection of *R. terrigena* TISTR 1568 in water with chlorine

The design points of the *R. terrigena* TISTR 1568 (log) used CCD arrangement and response for disinfection of *R. terrigena* TISTR 1568 in water with chlorine were shown in table 2. In tables 3 and 4, statistical analysis of the model was performed to determine the variance (ANOVA) for the quadratic polynomial mode land linear regression coefficients indicated that were obtained by employing a least square technique to predict quadratic polynomial model for *R. terrigena* TISTR 1568 (log).

The characteristics of the models and the coefficients indicated that the predictability of the model was at 95% confidence level. The model had high correlation coefficient, a significant F-value, an insignificant lack-of-fit F-value. The analysis of variance (ANOVA) showed that the model F-values was 10.177 with significance of 0.001. This significance of 0.001 was less than 0.05 for the significant models. So it implied the model was significant. The high correlation coefficient ($R^2 = 0.902$) indicated that the model was suitable to represent the real relationships among the parameters studied.

The insignificant lack-of-fit which is relative to the pure error of the experiments also indicates that the models were suitable to represent the experimental data. Coefficients of full model equation were evaluated by regression analysis and tested for their significances. The models to predict the *R. terrigena* TISTR 1568 (log) of disinfection of *R. terrigena* TISTR 1568 in water with chlorine was presented in equation (2).

$$Y = 17.817-9.990C-5.162P+0.072T+4.946C^2+0.524P^2+0.000T^2-0.257CP-9.814E-17CT-0.013PT$$

(2)

Table 2. ANOVA for the quadratic polynomial model of disinfection of *R. terrigena* TISTR 1568 in water with chlorine.

| Model   | Sum of squares | df | Mean square | F        | Sig.    |
|---------|----------------|----|-------------|----------|---------|
| Regression | 129.256 | 9  | 14.362      | 10.177   | 0.001*  |
| Residual | 14.112 | 10 | 1.411       |          |         |
| Total   | 143.367 | 19 |             |          |         |

* Predictors: (Constant), PT, CC, PP, CT, TT, CP, T, C, P.
Table 3. CCD arrangement and response for disinfection of *R. terrigena* TISTR 1568 in water with chlorine.

| Treatment | C | P | T | C (ppm) | P (min) | TIR TISTR 1568 (log) | Experimental | Predicted |
|-----------|---|---|---|---------|---------|----------------------|--------------|-----------|
| 1         | -1| -1| -1| 0.50    | 5.50    | 11.00                | 0.00         | 0.82      |
| 2         | -1| -1|  1| 0.50    | 5.50    | 39.00                | 0.00         | 0.83      |
| 3         | -1|  1| -1| 0.50    | 8.50    | 11.00                | 6.34         | 6.52      |
| 4         | -1|  1|  1| 0.50    | 8.50    | 39.00                | 5.23         | 5.45      |
| 5         |  1| -1| -1| 1.50    | 5.50    | 11.00                | 0.00         | -0.69     |
| 6         |  1| -1|  1| 1.50    | 5.50    | 39.00                | 0.00         | -0.68     |
| 7         |  1|  1| -1| 1.50    | 8.50    | 11.00                | 5.57         | 4.24      |
| 8         |  1|  1|  1| 1.50    | 8.50    | 39.00                | 4.46         | 3.16      |
| 9         | -1.68| 0 |  0| 0.16    | 7.00    | 25.00                | 6.78         | 5.12      |
| 10        | +1.68| 0 |  0| 1.84    | 7.00    | 25.00                | 0.00         | 1.94      |
| 11        |  0| -1.68| 0 | 1.00    | 4.48    | 25.00                | 0.00         | -0.64     |
| 12        |  0| +1.68| 0 | 1.00    | 9.52    | 25.00                | 6.45         | 7.38      |
| 13        |  0|  0| -1.68| 1.00    | 7.00    | 1.48                 | 0.00         | 0.49      |
| 14        |  0|  0| +1.68| 1.00    | 7.00    | 48.52                | 0.00         | -0.41     |
| 15        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |
| 16        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |
| 17        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |
| 18        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |
| 19        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |
| 20        |  0|  0|  0| 1.00    | 7.00    | 25.00                | 0.00         | 0.04      |

Table 4. Regression coefficients of predicted quadratic polynomial model for the regression equation of disinfection of *R. terrigena* TISTR 1568 in water with chlorine.

| Model     | Unstandardized Coefficients | Standardized Coefficients | t     | Sig. |
|-----------|-----------------------------|---------------------------|-------|------|
| 1 (Constant) | 17.817                      | 8.993                      | 1.981 | 0.076 |
| C         | -9.990                      | 4.931                      | -1.541| -2.026| 0.070 |
| P         | -5.162                      | 2.101                      | -2.389| -2.457| 0.034 |
| T         | 0.072                       | 0.174                      | 0.309 | 0.413 | 0.689 |
| CC        | 4.946                       | 1.254                      | 1.576 | 3.945 | 0.003 |
| PP        | 0.524                       | 0.139                      | 3.414 | 3.760 | 0.004 |
| TT        | 0.000                       | 0.002                      | 0.041 | 0.114 | 0.912 |
| CP        | -0.257                      | 0.560                      | -0.305| -0.458| 0.657 |
| CT        | -9.814E-17                  | 0.060                      | 0.000 | 0.000 | 1.000 |
| PT        | -0.013                      | 0.020                      | -0.432| -0.660| 0.524 |
3.2 Response Surface Plots of disinfection of $R. \text{terrigena}$ TISTR 1568 in water with chlorine

The optimized levels of variables were determined by constructing three-dimensional surface plots according to equation (2). The figure 1(a) shows the effect of chlorine concentration (C), pH (P) on the $R. \text{terrigena}$ TISTR 1568 (log) and their mutual interaction on the $R. \text{terrigena}$ TISTR 1568 (log) in water. The figure 1(b) shows the effect of chlorine concentration, disinfection time (T) on the $R. \text{terrigena}$ TISTR 1568 (log) and the figure 1(c) shows the effects of pH and disinfection time on the $R. \text{terrigena}$ TISTR 1568 (log). The figure 1(a), 1(b), 1(c) presented the limit values of the optimum values in disinfecting the $R. \text{terrigena}$ TISTR 1568 to 99.9% death. They indicate that pH is 4.48-7.5 and 1.48-45 min of disinfection time. Chlorine concentration should be used between 0.6 to 1.7 ppm which is obtained from the RSM.

![Response surface plots](image1.png)

Figure 1. Response surface plots representing the effect of factors on disinfection of $R. \text{terrigena}$ TISTR 1568 in water with chlorine predicted from the quadratic polynomial model.

3.3 Model Development for disinfection of $R. \text{terrigena}$ TISTR 1568 in water with chlorine

The model was developed to predict optimization of disinfection of $R. \text{terrigena}$ TISTR 1568 in water with chlorine by using optimization function of the SPSS software. The five conditions were selected from the optimum values in disinfecting the $R. \text{terrigena}$ TISTR 1568 to 99.9% death from response surface plot figure 1. They were both experimental values and predicted values which indicated that the comparison of both value was good correspondence between them, the empirical models derived from RSM can be used to describe the relationship between the factors and response in disinfection of $R. \text{terrigena}$ TISTR 1568 in water with chlorine. The prediction of the developed model showed that the optimized condition at 1.2 ppm of chlorine concentration, at pH 7 for 5 min of disinfection time.

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could disinfect the *R. terrigena* TISTR 1568 to 99.9% death. This condition was selected as the optimum condition because it could disinfect the *R. terrigena* TISTR 1568 to 99.9% death in minimum disinfection time. According to the definition, bactericidal activity is defined as a reduction of 99.9% (≥ 3 log10) of the total number of CFU/mL in the original inoculum [8].

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