

Research Article

Effect of micro-scale enrichment parameters on *Salmonella* growth in suspended cell cultivation

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Abstract

The conventional means for *Salmonella* identification is contingent upon rigorous culture-based methods. Suboptimal cultivation conditions and media type could play a crucial role in limiting *Salmonella* growth and undermining the effectiveness and sensitivity of the protocol. This study aims to optimize *Salmonella* cultivation conditions in a micro-scale environment; i.e., medium type, incubation temperature and initial cell loading to enhance the resolution of *Salmonella* detection protocol. The micro-scale cultivation was proposed to scale down the cultivation volume to 150 μ L. The turbidity of cultivation liquid representing *Salmonella* growth was observed using a microplate reader. Trypticase soy broth (TSB) and lactose broth (LB) were used as non-selective media, whereas Rappaport-Vassiliadis (RV) and Selenite Cystine (SC) were used as selective media. The logistic model applied to estimate growth kinetics performed well in describing the sigmoidal nature of batch cultivation of *Salmonella* in microwells. The maximum specific growth rates were then utilized to contrast different conditions of micro-scale cultivation. The TSB medium was found to be more preferable for *Salmonella* growth in the pre-enrichment and the differences between the RV and SC media were marginal. The optimal incubation temperature for both non-selective and selective enrichment was in the range of 37.5 to 40.0°C and the proper initial cell loading for detection using the microplate reader was between 10^5 and 10^7 CFU/mL due to equipment restrictions and time lag limitations.

Keywords: food contamination, growth parameter, *Salmonella*, microscale cultivation, TSB, LB, RV, SC, Thailand

Introduction

Among critical food-borne pathogens, *Salmonella* is widely considered as one of the most serious pathogenic contaminants and causes frequent disease outbreaks in many countries [1, 2]. *Salmonella* can cause illness ranging from gastroenteritis (e.g., abdominal cramp and diarrhea) to typhoid and paratyphoid fevers [3]. Occasionally, the infection leads to a life threatening situation or septicemia, particularly patients with impaired immune systems or immune deficiency. *Salmonella* contamination in food can be straightforwardly diagnosed by culturing the infected samples in a laboratory. However, high analytical cost and lengthy testing time established by most universally-accepted protocols discourage the exploitation of this conventional protocol for routine and quality assurance inspections [4]. This analytical complication paves the way for industrial microbiologists to develop more accurate and rapid methods for *Salmonella* detection.

Many food industries are trying to control the contamination of *Salmonella* and performing analytical tests for thorough process inspection. A few conventional standard methods (e.g., Bacteriological Analytical Manual (BAM), laboratory guidebook of Food Safety and Inspection Service (FSIS) and official method of Health Canada protocols) are generally applied for routine inspection. These methods are highly sensitive and selective for many target bacteria; however, they introduce analytical time restriction requiring around 3 to 5 days just having presumptive results. In some cases, it does also call for additional 1 to 2 days for confirmation using specific procedures [1, 4]. An efficiency improvement of these protocols is highly preferable while enabling the same or better accuracy, sensitivity and selectivity [3, 5].

The cultivation volume reduction can essentially optimize the use of reagent and media consumption, biohazard waste production and material requirement [6, 7]. In addition, micro-scale culturing technique can further enhance experimental controllability and repeatability as well as facilitating the transformation to fully-automatic robot machine [8]. To shorten *Salmonella* detection time, one of the key factors is to expedite the process of suspended-cell multiplication (both non-selective pre-enrichment and selective enrichment). This research aims to optimize the suspended cell cultivation and investigate the effect cultivation parameters (i.e., medium type, incubation temperature and initial cell loading) on cell growth and growth kinetics of *Salmonella*. The knowledge of *Salmonella* growth in a micro-scale environment may help lay the ground work for improving the sensitivity and resolution of microanalytical detection of *Salmonella* contamination as well as other food-borne pathogens.

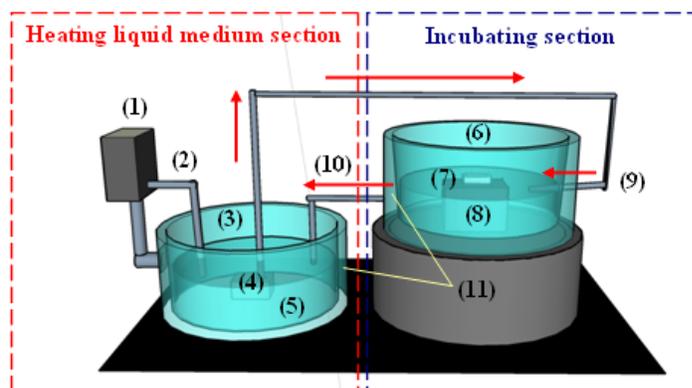
Materials and Methods

Salmonella strain preparation

Glycerol stock of *Salmonella anatum* culture (from Department of Medical Science, Ministry of Public Health, Thailand) was kept at -20°C and thawed at 37°C for 1 hr prior to use. *Salmonella* inoculum (100 µL) was recovered in 100 mL of TSB medium (Difco, USA) at 37°C for 7 h. Liquid culture (4 mL) was harvested and centrifuged at 3600 rpm for 20 min at 25°C. Cell pellets were re-suspended using normal saline solution (0.9% NaCl). The desirable initial cell number of *Salmonella* (i.e., 10^7 , 10^6 , 10^5 , 10^3 , 10^2 and 10^1 CFU/mL) were prepared via serial dilution. The number of viable cells was determined by a viable aerobic plate count method.

Sample preparation

Several cultivation parameters (i.e., medium type, incubation temperature and initial cell loading) were investigated. Three replicates of each micro-scale cultivation condition were performed in non-selective media, namely TSB and LB (Hi-media, India) media, and selective media, namely RV (Difco, USA) and SC (Hi-media, India). The experiments conditions were set in a 96-microwell plate; *Salmonella anatum* culture at various initial cell density (i.e., 10^7 , 10^5 , 10^3 , 10^2 , and 10^1 CFU/mL) were inoculated to those prepared media. The final volume of the cultivation was 150 μ L. A temperature-controlled water bath (Fig. 1) was fabricated to improve the accuracy of incubation temperatures (i.e., 35.0, 37.5, 40.0, 42.5 and 45.0°C)



- | | |
|----------------------------|---------------------------------------|
| (1) Temperature controller | (7) 96-microwell plate |
| (2) Thermocouple | (8) 96-microwell plate stand and jigs |
| (3) Water bath | (9) Heated liquid medium inlet |
| (4) Centrifugal pump | (10) Over flow outlet |
| (5) Hot plate (heater) | (11) Insulators |
| (6) Incubating chamber | |

→ : flow direction of heating medium

Figure 1. Schematic diagram of temperature-controlled water bath incubator.

A microplate reader (Varian Cary 50 MPR, Varian, Inc., USA) was used to measure the optical density at 600 nm wavelength for TSB, LB and SC media and 520 nm wavelength for RV medium. The difference of the optical density at time t (OD_t) and the initial optical density (OD_0) was utilized to observe the increase of *Salmonella* biomass. The profiles of the optical density changes were investigated as a function of time, medium type, incubation temperature and initial cell loading. The growth kinetics and characteristics of *Salmonella* in micro-environment, suspended cell cultivation were estimated using the logistic model.

Estimation of the maximum specific growth rate (μ_{max}) using logistic model

The logistic models were successfully used to describe and predict microbial growths in batch cultivation [9, 10]. The growth characteristics (e.g., maximum specific growth rate and lag time) of bacteria at specific environmental conditions can be calculated as a function of time using curve-fitting approach [11, 12]. The optical density or absorbance of inoculated media in an array of microwells was used to represent the *Salmonella* growth. Sigmoidal nature of *Salmonella* growth was captured using the logistic model as described in Equation 1.

$$\frac{dN(t)}{dt} = r(T)N(t) \left[1 - \frac{N(t)}{N_{\text{asympt}}(T)} \right] \quad (1)$$

Where $N(t)$ is a cell concentration at time t ; $r(T)$ is a temperature-dependent rate constant; and $N_{\text{asympt}}(T)$ is the asymptotic cell concentration.

The analytical solution of the cell concentration as a function of time can be shown as in Equation 2. This non-transformed, four-parameter logistic model normally provides a good fit to bacterial growth comparing to the Richards model, the modified Gompertz model and the Exponential model [14].

$$OD_t = OD_{\min} + \frac{OD_{\max} - OD_{\min}}{1 + \exp[-\mu_{\max}(t - t_i)]} \quad (2)$$

Where OD_t is optical density at time t ; OD_{\min} and OD_{\max} is the asymptotic minimum and maximum cell concentration in unit of absorbance (mAU), respectively; μ_{\max} is the maximum specific growth rate; t is an incubation time; and t_i is a time at inflection point (min).

The profiles of optical density data from various incubation conditions (i.e., medium type and incubation temperature used) were fitted to the above equation and numerically solved to determine the maximum specific growth rate and maximum cell concentration.

Statistical analysis

The designed experiment was completely random. The data were investigated using the analysis of variance (ANOVA), a statistical program MINITAB version 15 was used to perform the calculation. Independent variables were initial cell concentrations (10^1 , 10^2 , 10^3 , 10^5 , and 10^7 CFU/mL), incubation temperatures (35.0, 37.5, 40.0, 42.5 and 45.0°C), media types (both non-selective and selective media). Dependent variables were optical density growth curve of *Salmonella* and growth characteristics (e.g., maximum specific growth rate: μ_{\max} , and lag time: λ). To observe the difference between factor levels, Tukey's multiple comparisons was selected. Mean values were considered at 95% confidential interval ($\alpha=0.05$). All pairwise were compared at significant level $P \leq 0.05$.

Results and Discussion

The spectrophotometric approach has been frequently utilized to describe cell density of suspended cell cultivation [10, 11, 12, 14]. Brewster (2003) demonstrated the application of optical density readings from a 96-microwell plate to estimate bacterial concentration using a simple microwell reader. Similarly, the optical density changes of *Salmonella* culture in an array of microwells were used in this research to explore how well *Salmonella* biomass multiplies in different growth conditions.

Effect of incubation temperature

An experimental setting was successfully developed to precisely control incubation temperatures of the liquid broth inside microwells. A better control of broth temperature was achieved by means of a conductive heat transfer from the heating liquid rather than that of the conventional hot-air-type incubation. The *Salmonella* growth kinetics assimilating different media was contrasted to differentiate the effect of the slight changes of incubation

temperatures in a 150 μL cultivation volume. The different incubation temperatures significantly affected the growth profiles and characteristics of *Salmonella* (Fig. 2). The growth profiles suggested that the optimal incubation temperatures were in the range between 37.5 and 40.0°C. The non-selective enrichment of *Salmonella* at 37.5°C is rather high in temperature than that of the recommendation of the BAM protocol at $35\pm 1^\circ\text{C}$ [15]; however, this temperature are frequently suggested by other well-established protocols (e.g., ISO recommendation, FSIS, etc.).

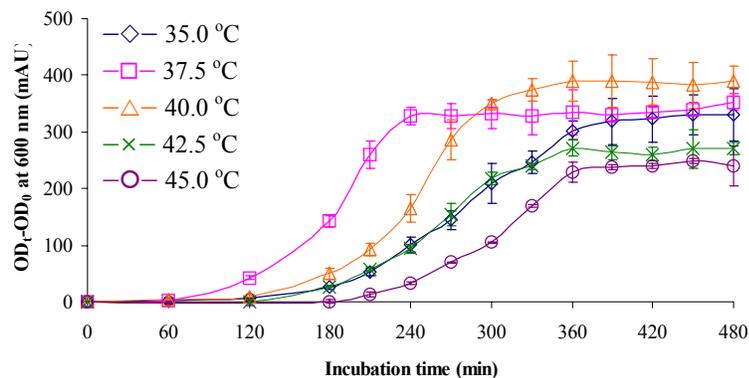


Figure 2. Experimental growth profiles of *Salmonella anatum* cultivation evaluated by the optical density differences using 150 μL TSB medium broth and 10^5 CFU/mL initial cell concentrations at various incubation temperatures.

A previous study by Membré et al. (2004) reported a larger collection of optimal temperatures from 35.0 to 44.0°C to enhance *Salmonella* growth. Up to 42.5°C was usually suggested for the selective cultivation. The application of high incubation temperatures was designed to improve the specificity to *Salmonella* growth by synergizing the effects between inhibitory agents in the selective media and the elevated temperature and to inhibit and screen out other non-*Salmonella* pathogens [16]. In this experiment, the growth profiles of *Salmonella* started to show an adverse effect of high incubation temperatures when the temperature of the medium exceeded 42.5°C.

Using the logistic model, the estimated maximum specific growth rate showed that the highest maximum specific growth rate of the non-selective TSB media was obtained at 37.5°C. The effect of the incubation temperatures on other standard media of *Salmonella* enrichment was also included. From Table 1, the non-selective LB medium was as good as or better in some cases than the TSB medium in enriching *Salmonella* samples reflecting by higher values of the maximum specific growth rate. For instance, the LB medium was more preferable to cultivate the bacteria at 40.0°C resulting in twice as much the maximum growth. In the selective SC medium, the selection of the incubation temperature also played a critical role in enhancing *Salmonella* growth. The best temperature for SC cultivation was at 37.5°C and this temperature was in the median of the suggested incubation range for selective enrichment recommended by major food regulation bodies (e.g., BAM, FSIS and Official Method of Health Canada at 35 ± 1 , 37 ± 1 , and $42.5\pm 0.5^\circ\text{C}$ respectively). Large fluctuations of the temperature from the optimal point could significantly worsen the growth characteristics of the bacteria.

Table 1. Comparison of the maximum specific growth rate: μ_{\max} (mAU/h⁻¹) of *Salmonella anatum* in 150 μ L batch cultivation using different types of media and incubation temperatures.

Incubation temperature (°C)	Media		
	TSB	LB	SC
35.0	28.8 ± 1.5 ^{A, a}	31.3 ± 2.9 ^{AB, a}	15.2 ± 2.0 ^{C, a}
37.5	40.2 ± 2.7 ^{A, b}	37.3 ± 3.4 ^{AB, ab}	23.2 ± 3.4 ^{C, b}
40.0	39.3 ± 1.4 ^{A, b}	47.9 ± 12.7 ^{AB, abc}	16.6 ± 1.3 ^{C, ac}
42.5	29.0 ± 1.4 ^{A, ac}	32.8 ± 3.0 ^{B, abcd}	17.2 ± 3.6 ^{C, abc}
45.0	27.0 ± 1.1 ^{A, d}	30.5 ± 5.2 ^{B, abcde}	15.0 ± 7.1 ^{C, abcd}

Note: Upper-case and lower-case letters designates row and column comparison, respectively. The means with the same superscript letters were statistically insignificant difference ($P \leq 0.05$). The means were averaged from three replicates.

Effect of medium type

It was evident that the non-selective group of media returned the higher optical density change comparing to the selective media (Fig. 3). The *Salmonella* treatment with TSB enrichment showed the best growth characteristics defined by short lag phase and distinct exponential growth. In contrast, the non-selective enrichment of *Salmonella* protocol described in the BAM recommendation frequently utilizes the LB medium for various kinds of food samples (e.g., meat, dried whole egg, fruit and vegetable). Optimization and selection of proper media in this pre-enrichment step can be critical to enhance the effectiveness of *Salmonella* detection since the sensible final cell number should be equal to or higher than 10^5 CFU/mL to resist *Salmonella* viable cells to selective agent toxicity [17].

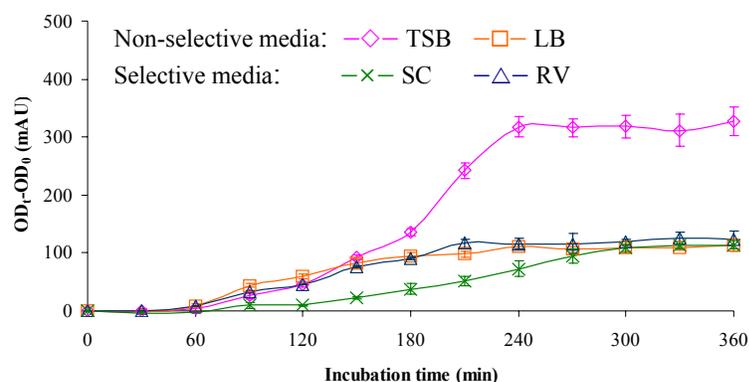


Figure 3. Experimental growth profiles of *Salmonella anatum* in different medium types at 37.5°C evaluated by the optical density differences using 150 μ L cultivation volume and 10^7 CFU/mL initial cell concentration.

Comparison of the profiles of the optical density increase suggested that the RV medium was more preferable to specifically raise the concentration of *Salmonella* in the selective enrichment step than the SC medium (Fig. 3). From the growth profiles, the RV medium was as effective in increasing biomass as the LB medium in the non-selective pre-enrichment. Generally, the selective media contain inhibitory agents that cause strong growth repression for the bacteria (e.g., enteric and gram positive bacteria) other than *Salmonella* [16]. The SC and RV media showed a significant inhibitory effect on *Salmonella* growth comparing to the non-selective media. Several authors also reported the different degrees of toxicity of

commonly-used selective media (e.g., tetrathionate brilliant green and Rappaport Vassiliadis) in *Salmonella* enrichment step [18, 19].

Effect of initial cell loading

The observability of *Salmonella* growth using this spectrophotometric methodology was highly dependent on the initial cell density in the medium. Higher initial cell concentrations in the TSB medium resulted in faster detection of turbidity increase (Fig. 4). Lower initial cell loadings extended the lag phase and prolonged the detectability of cell growth.

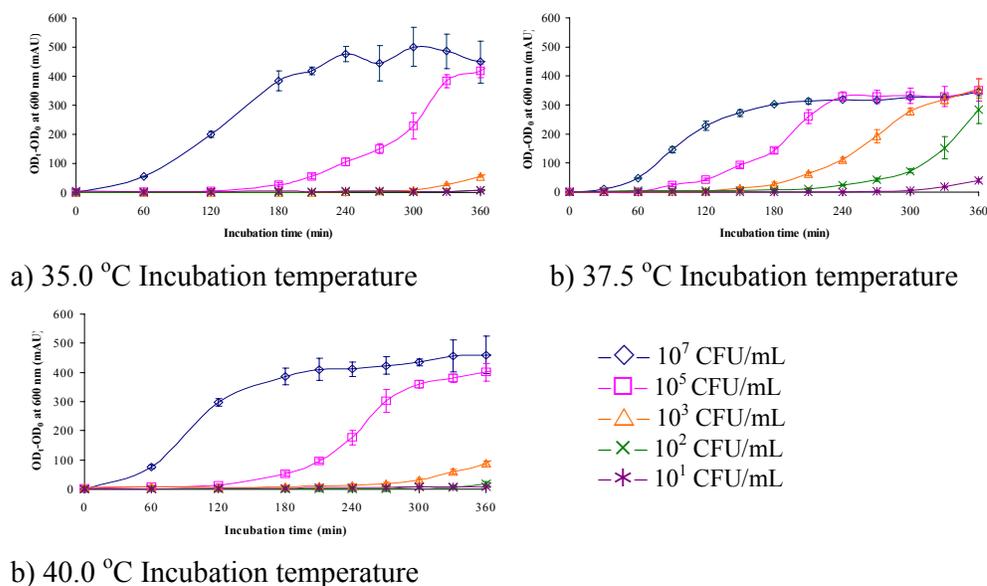


Figure 4. Experimental growth profiles of *Salmonella anatum* evaluated by the optical density differences using 150 μ L TSB medium broth at various initial cell concentrations.

The logistic model was used to estimate a few key growth characteristics from these profiles (Table 2). The differences of the maximum growth rates from each treatment were insignificant ($P > 0.05$). The initial cell loading only lengthened the lag phase having no effect on the growth characteristics [10, 15]. McKellar and Lu (2004) also recommended that the spectrophotometric detection of bacterial growth in suspended cell culture was appropriate when the minimum cell number was higher than 10^6 CFU/mL and the maximum cell density did not exceed 10^9 CFU/mL in most growth media.

Table 2. Mean values of maximum specific growth rate; μ_{\max} (mAU/h⁻¹) of *Salmonella* spp. at various initial cell loadings cultivated in TSB at 37.5°C.

Initial cell concentration (CFU/mL)				
10 ⁷	10 ⁵	10 ³	10 ²	10 ¹
Maximum specific growth rate: μ_{\max} (mAU/min)				
38.8 ± 2.9 ^A	40.2 ± 2.7 ^{AB}	37.7 ± 4.8 ^{ABC}	45.3 ± 6.1 ^{ABCD}	38.2 ± 2.6 ^{ABCD}
Lag time: λ (min)				
0 ± 0.0 ^A	185.4 ± 6.3 ^B	273.2 ± 17.7 ^C	331.7 ± 17.7 ^D	436.3 ± 1.6 ^E

Note: The means with the same superscript letters were statistically insignificant difference ($P < 0.05$). The means were averaged from three replicates.

Conclusions

The developed water-bath-type incubator enabled good control of the incubation temperature of liquid broth in microwells. The growth profiles measured from the turbidity changes were recorded and used to estimate the maximum specific growth rate using the logistic model. This mathematical model provided a good fit of the *Salmonella* growth data covering lag, exponential and stationary phases. The optimal incubation temperatures of *Salmonella* in micro-scale cultivation were in the range between 37.5 to 40.0°C, which were in the range recommended by most universal protocols (i.e., BAM, FSIS etc.). Significant alternation from this optimal point could result in substantial effects on the intrinsic growth kinetics. TSB medium was slightly more effective than the LB medium in boosting cell density for non-selective enrichment step. The RV medium also shows a slight advantage in multiplying *Salmonella* biomass in the selective enrichment. Using optical density to represent cell biomass was subjected to the sensitivity of the microplate reader. For this experiment, the growth profiles were observed when the cell concentration was equal to or higher than 10⁷ CFU/mL. This experiment helped shed light on the application of micro-scale cultivation to accelerate growth kinetics and facilitate the *Salmonella* detection method for food industry.

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