

Isolation of *Salmonella* from Natural Sources Representing High Potential for Biofilm Formations

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Abstract

Salmonella is a common food-borne pathogen that causes the deadly salmonellosis in humans and animals. *Salmonella* has been known to develop biofilm which protects it against antimicrobial agents and sanitizing agents on varieties of surfaces. In this study, seven *Salmonella* isolates from chicken intestine and egg from the markets in Bangkok illustrated the potential in developing biofilms on glass and polystyrene or plastic materials while they preferred to attach to the glass surface rather than the plastic surface. The isolates from chicken intestine illustrated the higher potential in developing the biofilm than the isolates from egg on both materials with the *P*-value of 0.0078 on polystyrene surface and 0.0218 on glass surface. One of the isolates from egg represented the lowest ability in biofilm formation than others.

Keywords: *Salmonella*, biofilm, chicken intestine and egg.

1. Introduction

Salmonella is a gram negative food-borne pathogen that causes salmonellosis disease in humans and animals. Salmonellosis causes an abdominal pain, inflammatory diarrhea and vomiting in the patient, which probably leads to death in some immune-compromised people. Humans obtain *Salmonella* through the consumption of contaminated animal products. Meat industry, dairy industry and poultry products are the principal reservoirs of salmonellae worldwide that mediate the disease transmission to the consumers (D'Aoust 1989). *Salmonella* persists in the factories due their abilities to develop biofilms on the surfaces of industrial equipments which are major sources of food contamination and disease transmission (Hood and Zottola 1997; Giaouris and Nychas 2006; Vestby 2009). Microbial colonization and embedding under extracellular polymeric substance (EPS), called 'biofilm', has been found in a wide range of environmental conditions or even in the nature (O'Toole *et al.* 2000). Biofilm commonly contaminates industrial pipelines, food contact surfaces, floors when an inappropriate sanitizing method has been applied in the industrial clean up since

the biofilm can develop on various kinds of surface materials (Chae and Schraft 2000). The presence of EPS of the mature biofilms causes the pathogens under the protective environment to be more tolerant to many stresses including the disinfectants or sanitizers than the planktonic cells or free floating cells (Gilbert *et al.* 2001).

Salmonella have abilities to form on glass, plastic, rubber and stainless steel materials in the industries (Ronner and Wong 1993; Carballo 2000). The biofilm of *Salmonella* was found to be more resistant to sanitizers than the planktonic cells. The routine cleaning with the chemical sanitizer in the food industry has a slight effect on the elimination of *Salmonella* mature biofilm (Mettler and Carpentier 1998). Therefore, *Salmonella* biofilms have a dramatic effect on the industrial clean up.

The purpose of this study was to screen out *Salmonella* from chicken intestine and egg from Bangkok's local markets using biochemical tests and study their potential for the formation of biofilms (Tammakritsada 2010). The biofilm developments of the isolates were tested using the crystal violet assay and direct microscopic method for the direct biofilm observation. Since the

Salmonella contamination in the industry is derived from natural reservoirs, therefore, the *Salmonella* isolates from the natural reservoirs that have high biofilm forming abilities will serve the future prospect in identifying the suitable strategy to eliminate *Salmonella* biofilms in the food industry.

2. Materials and Methods

2.1 Sample Collection and Pre-enrichment Method

Chicken intestine and egg were collected from the markets in Bangkok. The samples were grinded or blended and left in peptone 0.1% (V/V) overnight. Five milliliters of the culture samples were transferred to 45 ml of the trypticase soy broth (TSB) and culture for one day. The pre-enrichment samples were diluted in 0.1% (V/V) peptone to the dilution of 10^{-4} and plated on *Salmonella* Shigella agar (SS agar). The positive colonies of *Salmonella* represented by colorless colonies with black centers were picked up for further biochemical tests.

2.2 Biochemical test

The gram stain, spore stain, motility, catalase, citrate, aerobic and facultative anaerobic tests were performed on the positive colonies that were selected from SS agar plate. *Salmonella* is explained by the Bergey's manual of determinative bacteriology as gram negative, rod shape, non-spore forming, motile, catalase positive, Simons citrate positive and facultative anaerobic.

2.2.1 Gram Stain: Each positive sample was smeared on the clean glass slide and heat fixed. The few drops of crystal violet were dropped on the fixed cells to stain for 1 minute. Then the slide was rinsed with clean water and decolorized with 95% ethanol. The slide was rinsed again with clean water and counterstained with Safranin for 1 minute. At the final step, the slide was rinsed with water and observed under a bright-field microscope. The positive colony represents the red staining which is gram negative when observed. *Salmonella* is gram negative bacteria so it stains red.

2.2.2 Spore Stain: Each positive sample was smeared on the glass slide and heat fixed. The slide was placed on the beaker of water that was placed on the hot plate and boiled by the steam from the boiling water from the beaker for 2-3 minutes. Then the sample on the slide was flooded with malachite green and cooled down. The stained slide was washed with water and counterstained with safranin for 1 minute and washed with the water again. The spores were detected under the microscope. The positive colony represented the non-spore forming with no greenish stain.

2.2.3 Motility Test: The motility test was performed on each positive sample to identify whether it was motile or non-motile. Each colony was stabbed on the 0.3% (W/V) TSA plate. The motile positive colonies showed to have a movement outward from the stabbing area after the incubation period of 24 hours at 37°C. The positive colony of *Salmonella* represented the motility positive ones.

2.2.4 Catalase Test: The catalase test was done on each positive sample by smearing a small amount of the organism onto the TSA plate and grew overnight at 37°C. Few drops of hydrogen peroxide (H_2O_2) were dropped to the smear. The positive colony of *Salmonella* represented the catalase positive one when the bubble became visible after dropping H_2O_2 .

2.2.5 Citrate Test: The positive sample was streaked on Simon's citrate agar slant. After the overnight incubation at 37°C, the positive colony of *Salmonella* represented the change of the medium color from green to blue.

2.2.6 Aerobic and Facultative Anaerobic Test: The positive sample was placed in TSA deep tube and overlaid with 1-cm thick glycerol. The culture was incubated for 24 hours at 37°C. The positive colony represented the growth under the test condition and in the absence of glycerol which meant to be a facultative anaerobe.

2.3 Identification of the Maximum Specific Growth Rate (μ_{max})

The maximum specific growth rate was identified for all the *Salmonella* samples (μ_{max} , $hour^{-1}$). Each sample was grown in 5 ml TSB

and transferred to 50 ml TSB shake flask at 120 rpm. The optical density was measured at a wavelength of 600 nm every hour. The experiment was performed in triplicate and the absorbance readings were averaged.

2.4 Quantitative Analysis on the Biofilm Formation

The quantitative analysis of each *Salmonella* strain on its biofilm forming ability or cell adhesion ability was performed using crystal violet staining of the attached cells. The quantitative assay on the biofilm formation was performed in glass and polystyrene tubes. Glass tube was prepared by rinsing with distilled water then air-dried and autoclaved at 121°C for 15 minutes. Polystyrene (PS) tube was prepared by soaking with 95% ethanol for 30 minutes. The total of 1% (v/v) of the bacterial culture ($OD_{600nm} \approx 1.0$) was inoculated into 2 ml of diluted 1:20 TSB (dTSB) since dTSB medium was reported as a suitable medium for *Salmonella* biofilm development (Stepanovic *et al.* 2004). Biofilms were allowed to develop under the static condition at 37°C. Biofilm development was identified on day 1, day 2 and day 3 by crystal violet staining (1% w/v) and spectroscopic determination. At each time point, the supernatants of the tubes were aspirated and the tubes were rinsed 3 times with distilled water and then fixed by drying on the bench top until they were fully dried out. The total of 2 ml of 1% crystal violet was added into each tube to stain for 25 minutes. The excess stain was washed off for 3 times with distilled water. The crystal violet that stained the attached cells was destained with 2 ml of 95% ethanol by leaving it at room temperature for 30 minutes. The optical density (OD_{595nm}) of the destained solution was examined using a spectrophotometer (Djordjevic 2002). The absorbance value was positively correlated to the amount of the bacterial adhesion or biofilm. All tests were performed in triplicate and the absorbance readings were averaged.

2.5 Visual Observation on the Biofilm Development

The test was carried out using the flat surface of glass slide. The flat glass surface used in this study had the dimension of 22 mm x 22 mm. The slide was prepared by rinsing with distilled water, air-dried and then autoclaved at 121°C for 15 minutes.

The biofilm of each strain was allowed to develop on the glass slide for 3 consecutive days by submerging the slides on the plates containing 20 ml of dTSB. The biofilms were allowed to develop at 37°C under the static condition. Fresh media were replaced every 24 hours. Daily sampling was carried out where each surface was carefully removed with sterile forceps and slightly air-dried in the laminar flow. The biofilm development in each condition was examined under the bright-field microscope with the magnification of 400x. Each experimental assay was performed in triplicate.

3. Results and Discussion

The purpose of this study was to screen out *Salmonella* from chicken intestine and egg and studied on their abilities to develop the biofilms. The samples were pre-enriched and cultured on SS agar as a selective medium for *Salmonella*. Seven candidates were isolated from the chicken intestine and egg samples in which four isolates from chicken intestine (isolate numbers 1-4) and three isolates from egg (isolate numbers 5-7). Seven positive isolates of *Salmonella* represented the colorless colony with the black center were picked up for the further biochemical test to confirm the result. From the biochemical test, all seven isolates represented the phenotypic characteristic of *Salmonella* (Table 1). The maximum specific growth rates (μ_{max}) and the doubling time of all isolates were identified based on the optical density at the wavelength 600 nm (Table 2). *Salmonella* isolated from chicken intestine represented the non significant difference on the maximum specific growth rate to the *Salmonella* isolated from egg in which the *P*-value was 0.8184.

Table 1. The biochemical tests of all seven isolates.

Isolate number	Origin	Gram stain (- gram positive)	Spor e	Motility	Catalas e	Citrate	Aerobic and facultative anaerobic
1	Chicken intestine	-	-	+	+	+	Facultative anaerobe
2	Chicken intestine	-	-	+	+	+	Facultative anaerobe
3	Chicken intestine	-	-	+	+	+	Facultative anaerobe
4	Chicken intestine	-	-	+	+	+	Facultative anaerobe
5	Egg	-	-	+	+	+	Facultative anaerobe
6	Egg	-	-	+	+	+	Facultative anaerobe
7	Egg	-	-	+	+	+	Facultative anaerobe

Table 2. The maximum specific growth rate (μ_{\max}) of all seven isolates.

Isolate number	Doubling time (t_d , hour)	Maximum specific growth rate (μ_{\max} , hour ⁻¹)
1	1.06	0.653
2	1.2	0.577
3	0.8	0.866
4	0.8	0.866
5	0.93	0.745
6	0.87	0.796
7	0.93	0.745

The quantitative analysis of biofilm formation has been analyzed in many microorganisms by using crystal violet staining the total attached biomass on the surface (O'Toole *et al.* 1999). Therefore, this application was used to analyze the biofilm formation of all seven *Salmonella* isolates. The biofilm formations of all isolates were studied on polystyrene as a representative of hydrophobic surface and glass as a representative of hydrophilic surface. Figure 1 demonstrates the biofilm formations as referred as bacterial attachment of all isolated *Salmonella*, which were obviously developed on the air-liquid interface in glass and polystyrene tubes. The biofilms were obviously seen after staining with crystal violet. As *Salmonella* is a facultative aerobic bacterium, the air-liquid interface could be a preferred area for the bacterial attachment that benefits the cells in terms of oxygen supply and nutrient accessibility from the top rather than the bottom of the tube (Fig. 1). Upon the quantitative assay

after destaining the attached cells which refer to biofilm with ethanol, the higher the OD_{595nm} intensity, the higher the biofilm formation. The biofilm formations of all strains were continuously increased along the cultivation period in which the optical density of dissolved crystal violet continuously increased from day 1 to day 3 in both materials (Fig. 2). The biofilm developments of all strains were shown to be significantly higher in glass surface than in polystyrene surface which could be interpreted that *Salmonella* isolated from natural reservoirs probably preferred to attach on the hydrophilic surface rather than on the hydrophobic surface. In both materials, the isolated strain number 7 represented the lowest biofilm formation in comparison to all strains despite the differences on the growth rates. The isolates from chicken intestine (no. 1-4) illustrated the higher potential in developing the biofilm than the isolates from egg (no. 5-7) in both materials. The *P*-values were 0.0078 on polystyrene surface and 0.0218 on glass surface when

compared the biofilm forming abilities of isolates from chicken intestine and egg which were considered to be statistically significant differences on both materials.

The further visual observation of biofilm formation on glass slide under the bright-field microscopic analysis was performed for 3 days of the biofilm development under the static condition of all isolates (Fig. 3). The number of the bacterial attachment increased continuously along 3 days of the experiment for all isolates. The extracellular polymeric substance seemed to be developed on day 3 in which the matrix was slightly formed. The lowest number of bacterial attachment was found on isolate No.7 on day 3 in comparison to other isolates on the same day in which it corresponded to the crystal violet assay where it represented the lowest intensity when compared to other isolates (Fig. 2).

4. Conclusion

In conclusion, it was shown that 4 isolated *Salmonella* from chicken intestine and 3 isolated *Salmonella* from egg were capable to form biofilms on glass and plastic materials while they preferred to attach on glass surface which is a hydrophilic surface. The direct observation represented the biofilm forming ability in correspondence to the indirect assay using crystal violet staining the attached cell in which the isolate No. 7 from egg illustrated the lowest biofilm forming ability. The isolates from chicken intestine illustrated the higher potential in developing the biofilm than the isolates from egg on both materials. This research provided the future prospect in searching for an effective strategy to eliminate *Salmonella* biofilm since this study indicated that *Samonella* is naturally able to live in the biofilm form even with the isolates from the natural reservoirs.

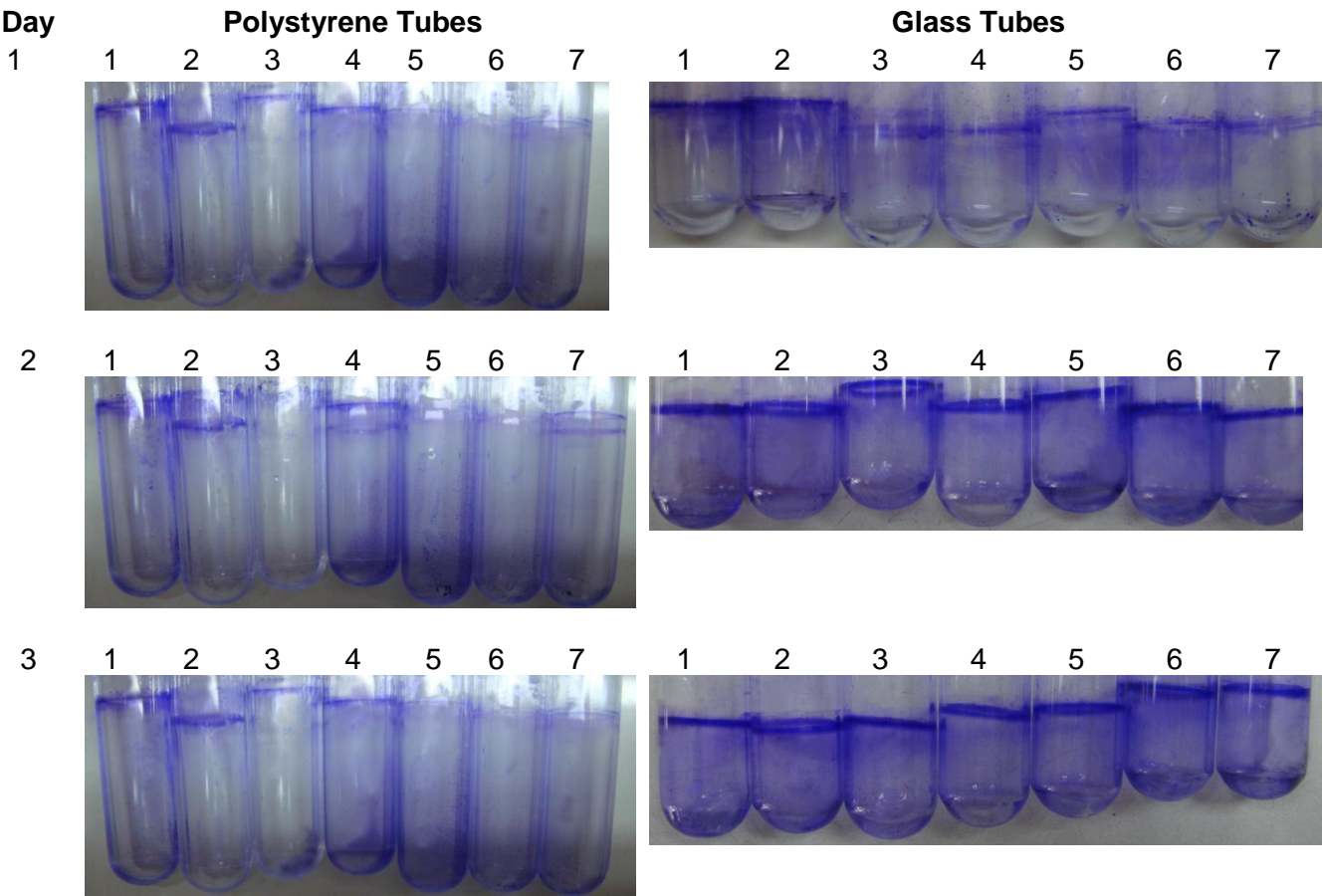
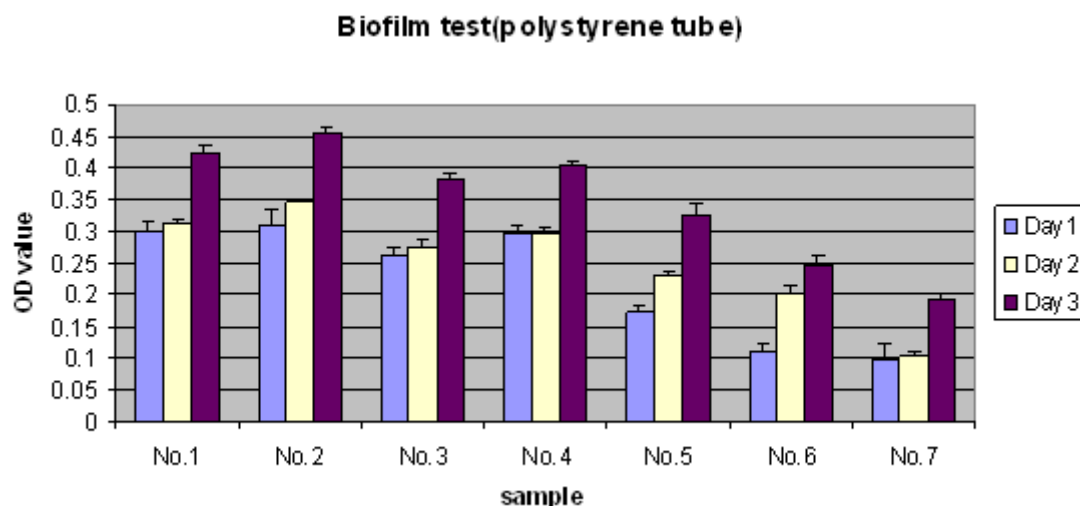
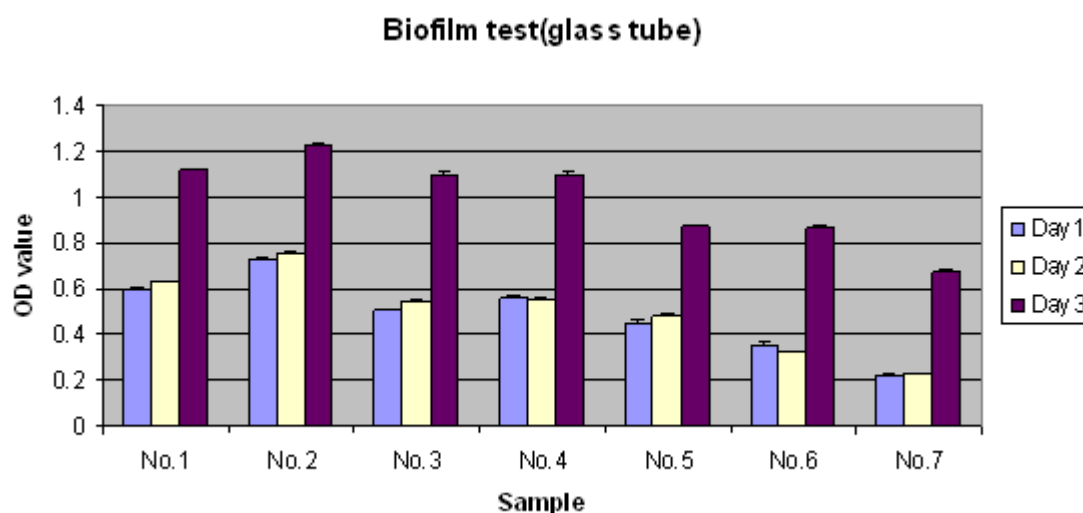


Fig. 1. Biofilm formations of all isolates on polystyrene tubes and glass tubes after staining with crystal violet solution (day 1 - day 3). The number above the picture represented the number of the isolate.



(a) Biofilm formation in polystyrene tube.



(b) Biofilm formation in glass tube.

Fig. 2. All the positive isolates (Number 1-7) were tested on the quantitative assay upon the biofilm formation on (a) polystyrene tube and (b) glass tube using crystal violet staining and de-stained with ethanol. The assay was conducted consecutively for 3-day cultivation period.

5. References

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Fig. 3. The figure represents the visual observation of the biofilm developments of all isolates under the bright-field microscope upon 3-day cultivation period on the glass surface. The pictures were captured with 400X magnification.

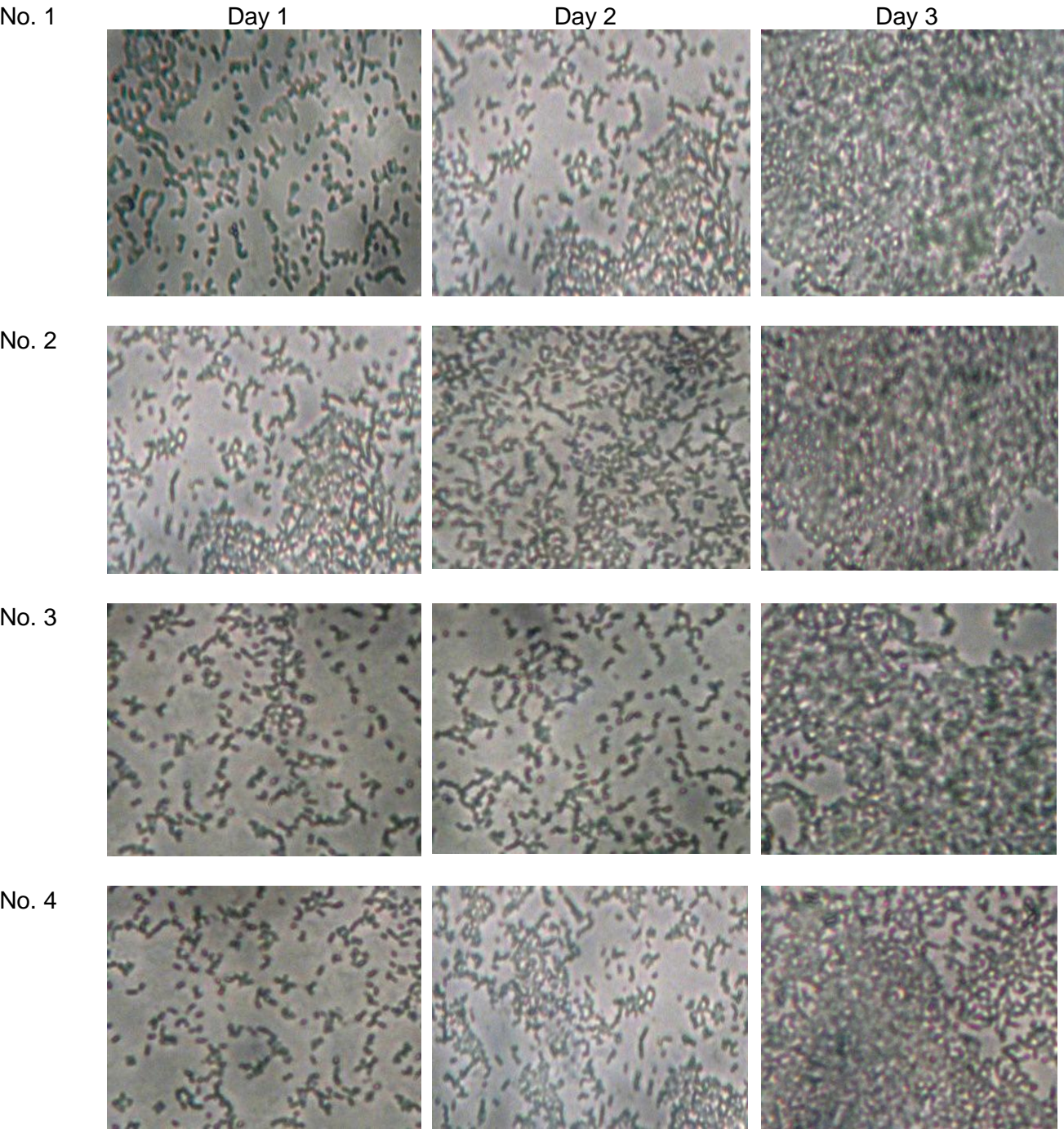
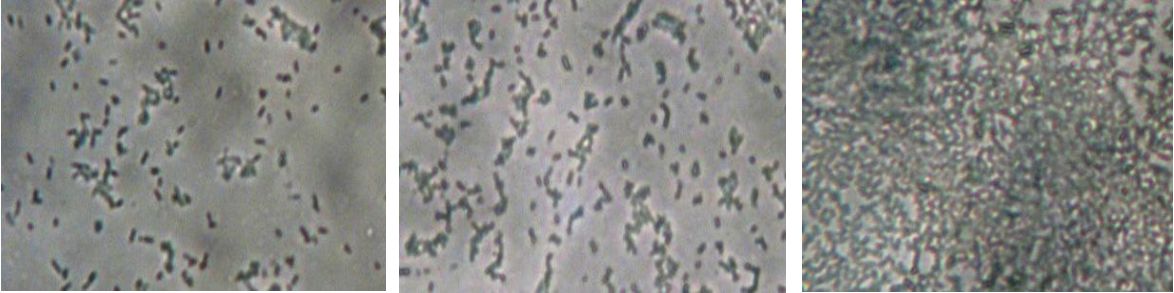
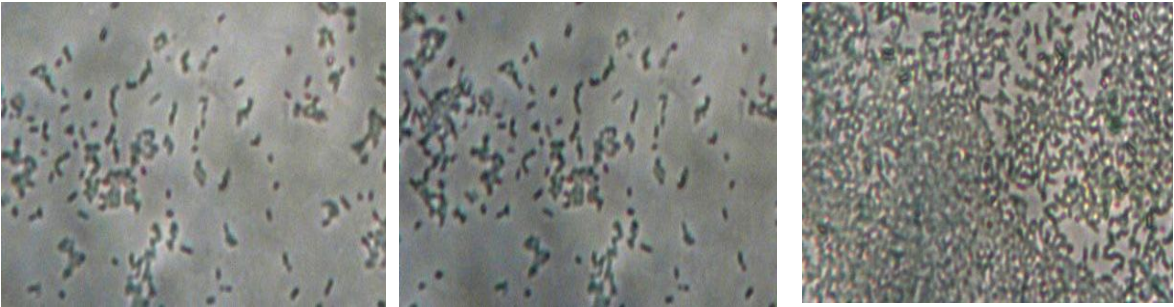


Fig. 3. (Continued)

No. 5



No. 6



No. 7

