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# Growth Characteristics and Biofilm Formation of Various Spoilage Bacteria Isolated from Fresh Produce

Young-Min Bae, Ling Zheng, Jeong-Eun Hyun, Kyu-Seok Jung, Sunggi Heu, and Sun-Young Lee

**Abstract:** This study investigated the characteristics of spoilage bacteria isolated from fresh produce including growth at various temperatures, biofilm formation, cell hydrophobicity, and colony spreading. The number of spoilage bacteria present when stored at 35 °C was significantly greater than when stored at lower temperatures, and maximum population size was achieved after 10 h. However, *Bacillus pumilus*, *Dickeya zeae*, *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc21, and *Bacillus pumilus* (RDA-R) did not grow at the storage temperature of 5 °C. The biofilm formation by *Clavibacter michiganensis*, *Acinetobacter calcoaceticus*, and *A. calcoaceticus* (RDA-R) are higher than other spoilage bacteria. Biofilm formation showed low correlation between hydrophobicity, and no significant correlation with colony spreading. These results might be used for developing safe storage guidelines for fresh produce at various storage temperatures, and could be basic information on the growth characteristics and biofilm formation properties of spoilage bacteria from fresh produce.

**Keywords:** biofilm, hydrophobicity, growth, spoilage bacteria, temperature

**Practical Application:** Growth of spoilage bacteria was different depending on the bacteria strains and storage temperature. Between biofilm formation and cell hydrophobicity was low correlation on spoilage bacteria. Therefore, growth characteristics and biofilm formation of spoilage bacteria might be used for developing safe storage guidelines for fresh produce at various storage temperatures.



## Introduction

The demand for minimally processed fresh vegetables has led to increases in the quantity and variety of products available for the consumer. Since fresh products are usually consumed raw, maintaining the shelf life of fresh products is important. Spoilage is characterized as any change in a food product that presents itself as unacceptable to the consumer from a sensory point of view. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), textural changes (degradation of polymers), or off-odors and off-flavors (NRC 1985). The growth and activity of spoilage bacteria is mostly described and studied as a function of substrate base, and physical or chemical parameters such as water activity, pH, temperature, and atmosphere (Lone and others 2002). Temperature control is an important factor in maintaining the quality and shelf life of fruits and vegetables (Smyth and others 1998). As microbial spoilage is a major component of food spoilage, the effect of temperature on the specific growth rate of microorganisms is important, especially in stores containing fresh produce. It is suggested that all refrigerated foods be stored at 5 °C or below by the Food and Drug Administration (FDA) uniform food code, because temperatures above this promote bacterial growth (Matthews 2006). However, in the process of fresh produce transport and marketing, it is difficult to maintain a constant temperature. Therefore, studying the behavior of spoilage bacteria at different temperatures and how spoilage bacteria adapt are important for preserving food quality.

Biofilms have been observed on various food and food processing surfaces, and can cause increasing risks for fresh product quality and public health (Carmichael and others 1999; Van Houdt and Michiels 2010; Liu and others 2013). Fresh products and food contact surfaces often have relatively rich nutrient concentrations, and together with suitable environmental conditions such as temperature, relative humidity, and pH, can promote biofilm formation (Chmielewski and Frank 2003; Fratamico and others 2009). In addition, attached microorganisms and biofilms formed by microorganism are not easily removed by washing with water, and are resistant to cleaning and disinfection processes in the food industry (Joseph and Otta 2001; Lapidot and others 2006). Biofilm formation is a complex process, which involves genetic mechanisms and numerous factors, such as the properties of the involved substratum and bacterial cell surfaces (Shi and Zhu 2009). Of the various factors affecting the attachment of bacterial cells to a surface such as hydrophobicity, surface charge, material properties, and mode of growth, hydrophobicity is a significant determinant of adhesion and biofilm formation on surfaces (Goulter and others 2010; Takahashi and others 2010). The attachment to the surface of fresh products may be considered as a 1st step in the microbial spoilage of vegetable and fruits; continued microbial presence and growth will depend on their ability to remain attached to the surfaces of fresh products (Benito and others 1997). The cell characteristics of spoilage bacteria, including biofilm formation, cell hydrophobicity, and colony spreading, is important for determining hygiene and shelf life of fresh products, either

*MS 20140899 Submitted 5/27/2014, Accepted 8/8/2014.* *Authors Bae, Zheng, Hyun, Lee are with Dept. of Food Science and Technology, Chung-Ang Univ., 72-1 Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi-do, 456-756, South Korea. Authors Jung and Heu are with Microbial Safety Div, Dept. of Agro-food Safety, Natl. Academy of Agricultural Science Rural Development Administration, Suwon, 441-857, Republic of Korea. Direct inquiries to author Lee (E-mail: nina6026@gmail.com or nina6026@cau.ac.kr).*




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to prevent or remove bacterial contamination. However, studies of biofilm attachment and cell hydrophobicity of spoilage bacteria have not been performed for a wide range of food spoilage bacteria.

Therefore, in this study, characteristics of spoilage bacteria isolated from fresh produce were examined at various storage temperatures, and then a predictive growth model (modified Gompertz equation) was used for confirmation of the spoilage bacteria growth in different storage temperatures. The biofilm formation ability, cell hydrophobicity, and colony spreading of the studied strains were also investigated.

## Materials and Methods

### Preparation of bacterial strains

Fifteen strains of spoilage bacteria (*Chryseobacterium balustinum*, *Pantoea agglomerans*-1, *Pantoea agglomerans*-2, *Bacillus pumilus*, *Clavibacter michiganensis*, *Pseudomonas fluorescens*-1, *Pseudomonas fluorescens*-2, *Stenotrophomonas maltophilia*-1, *Stenotrophomonas maltophilia*-2, *Klebsiella pneumonia* were isolated from lettuce, *Acinetobacter calcoaceticus*-1, *Acinetobacter calcoaceticus*-2 were isolated from chicory, and *Dickeya zeae*, *Enterobacter* sp., and *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc21 were isolated from Chinese cabbage) isolated from fresh produce were obtained from Rural Development Administration of Korea (RDA), and used in this study (Lee and others 2013). Also, 7 rifampicin-resistant strains (RDA-R; *P. agglomerans*, *D. zeae*, *A. calcoaceticus*, *S. maltophilia*, *K. pneumonia*, *B. pumilus*, *C. michiganensis*) were used. All strains were maintained at −80 °C in 20% glycerol and were activated by cultivation in tryptic soy broth (TSB, pH 7.3) for 24 h at 37 °C before use.

### Measurement of spoilage bacterial growth

At each temperature (5, 15, 25, and 35 °C), 0.1 mL of the spoilage bacteria was inoculated in 5 mL sterile TSB, and then incubated. The optical density (OD) at 595 nm of an 8 mL tube

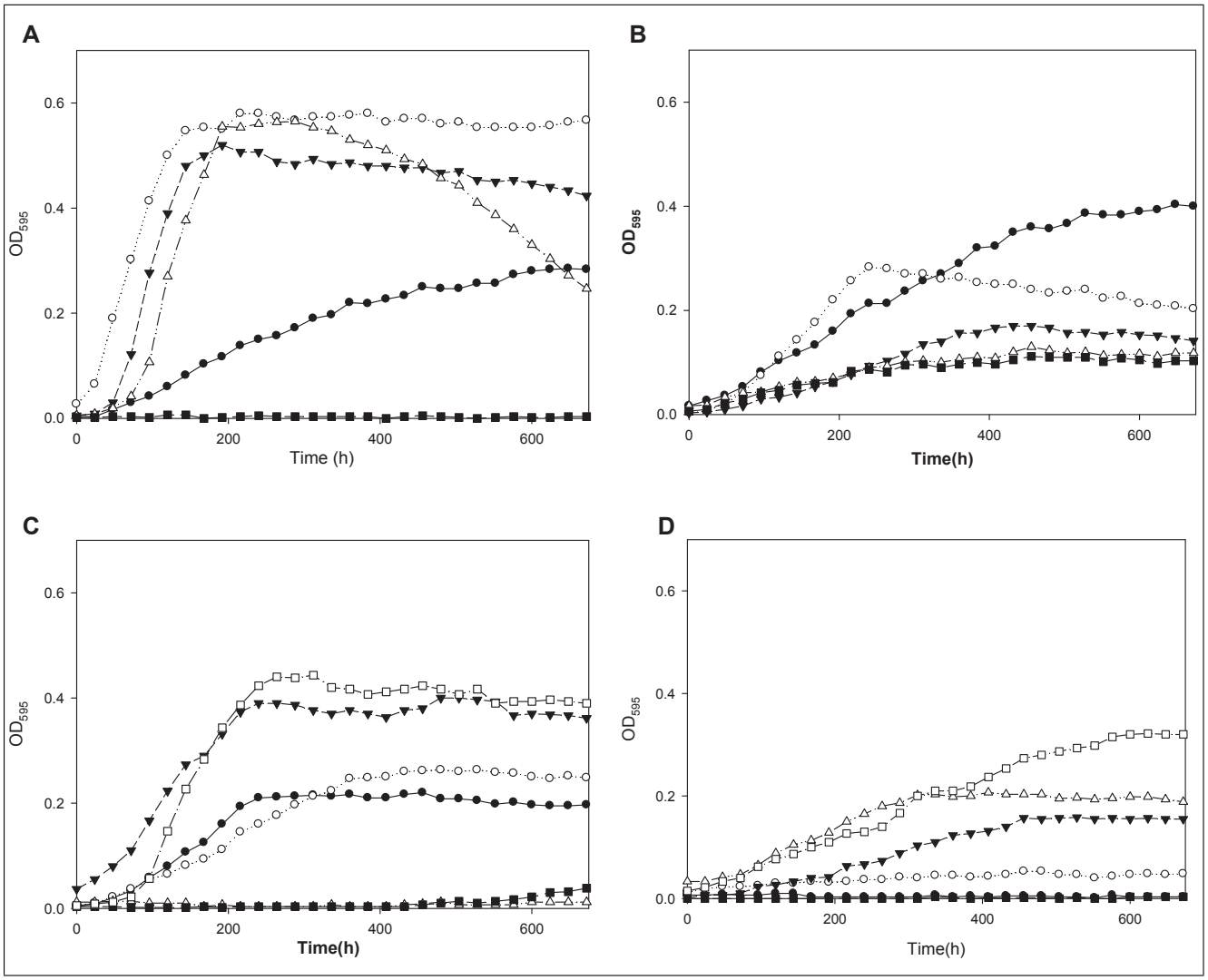



Figure 1–Growth of 22 spoilage bacteria isolated from fresh produce at 5 °C.
(A) ●, *Chryseobacterium balustinum*; ○, *Enterobacter* sp.; ▼, *Pantoea agglomerans* -1; △, *Pantoea agglomerans*-2; ■, *Bacillus pumilus*. (B) ●, *Clavibacter michiganensis*; ○, *Pseudomonas fluorescens*-1; ▼, *Pseudomonas fluorescens*-2; △, *Acinetobacter clacoaceticus*-1; ■, *Acinetobacter clacoaceticus*-2. (C) ●, *Stenotrophomonas maltophilia*-1; ○, *Stenotrophomonas maltophilia*-2; ▼, *Klebsiella pneumonia*; △, *Dickeya zeae*; ■, *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc2; □, *Pantoea agglomerans* (RDA-R). (D) ●, *Dickeya zeae* (RDA-R); ○, *Acinetobacter clacoaceticus* (RDA-R); ▼, *Stenotrophomonas maltophilia* (RDA-R); △, *Klebsiella pneumonia* (RDA-R); ■, *Bacillus pumilus* (RDA-R); □, *Clavibacter michiganensis* (RDA-R).

was determined using a Spectronic 20 spectrophotometer (Miltomroy Co., Ivyland, Pa., U.S.A.). The spectrophotometer was zeroed using a sterile tube with 5 mL sterile TSB. Growth was measured every hour during one day of incubation at 15, 25, or 35 °C, and then measured every 24 h. The growth observed at 5 °C was measured every 24 h. Every test was performed in triplicate.

## Growth modeling

Growth data were iteratively fit to a modified Gompertz equation using a nonlinear regression model (Prism, version 4.0, GraphPad Software, San Diego, Calif., U.S.A.) to determine growth rates (GR, $h^{-1}$) and lag time (LT, h) at each incubation temperature. The following modified Gompertz equation, which was described by Gibson and others (1988), was used.

$$Y = N_0 + C \times \exp[-\exp\{(2.718 \times \mu/C) \times (\mathrm{Lag} - X) + 1\}],$$

where $Y$ is the log cell number ($\log_{10}$CFU/g), $X$ is the incubation time (h), $N_0$ is the log initial number of cells ($\log_{10}$CFU/g), $C$ is the difference between initial and final cell numbers ($\log_{10}$CFU/g), Lag is the lag time before growth (h), and $\mu$ is the maximum specific growth rate ($\log_{10}$CFU/h).

## Biofilm formation

Each strain of spoilage bacteria isolated from fresh produce was cultured in TSB for 24 h at 37 °C. To form bacterial microtiter plate biofilm, wells of sterile 96-well polystyrene plates were filled with 90 $\mu$L TSB and inoculated with 10 $\mu$L of spoilage bacteria. Negative control wells containing only TSB were included for each assay. The inoculated bacteria were incubated at 37 °C for 24 h. After discarding the medium in the microtiter plate wells by inversion, wells were rinsed 3 times with distilled water (200 $\mu$L/well). After air drying, wells were stained with 50 $\mu$L of 0.5% crystal violet for 10 min. Excess stain was removed by washings



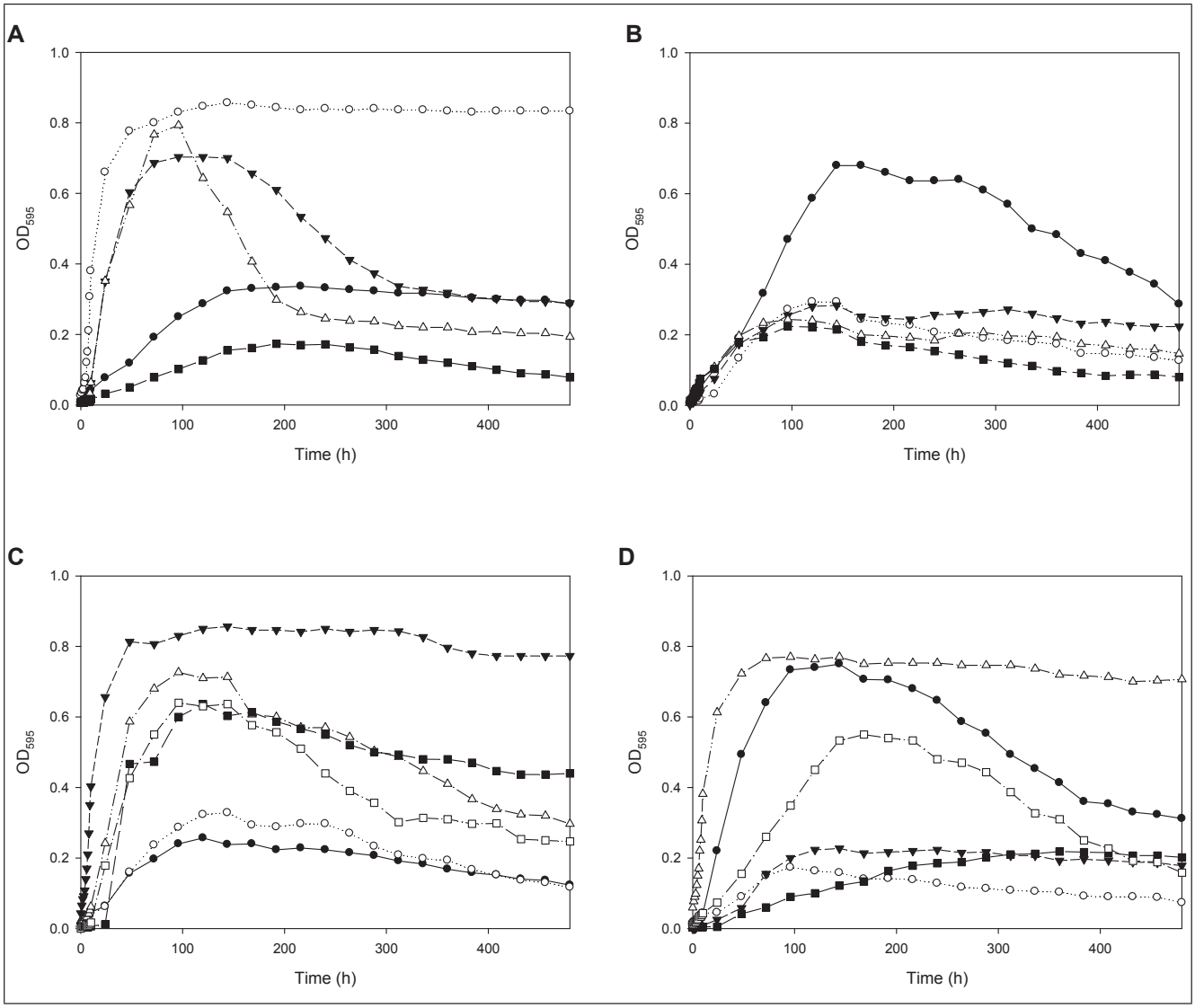



Figure 2–Growth of 22 spoilage bacteria isolated from fresh produce at 15 °C.
(A) ●, *Chryseobacterium balustinum*; ○, *Enterobacter* sp.; ▼, *Pantoea agglomerans*-1; △, *Pantoea agglomerans*-2; ■, *Bacillus pumilus*. (B) ●, *Clavibacter michiganensis*; ○, *Pseudomonas fluorescens*-1; ▼, *Pseudomonas fluorescens*-2; △, *Acinetobacter clacoaceticus* -1; ■, *Acinetobacter clacoaceticus*-2. (C) ●, *Stenotrophomonas maltophilia*-1; ○, *Stenotrophomonas maltophilia*-2; ▼, *Klebsiella pneumonia*; △, *Dickeya zeae*; ■, *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc2; □, *Pantoea agglomerans* (RDA-R). (D) ●, *Dickeya zeae* (RDA-R); ○, *Acinetobacter clacoaceticus* (RDA-R); ▼, *Stenotrophomonas maltophilia* (RDA-R); △, *Klebsiella pneumonia* (RDA-R); ■, *Bacillus pumilus* (RDA-R); □, *Clavibacter michiganensis* (RDA-R).

with distilled water (200 $\mu$L/well). Dye bound to adherent cells was destained with 50 $\mu$L of 99% ethanol, and the OD of each well was obtained at 595 nm using a spectrophotometer (Specronic® 20 Genesys, Spectronic Instruments, New York, N.Y., U.S.A.; Christensen and others 1985; Knowles and Roller 2001).

## Bacterial cell hydrophobicity

The bacterial adherence to hydrocarbons (BATH) method was performed as described by Goulter and others (2010) with minor modifications. Test strains were harvested at the stationary phase, collected by centrifugation (8000 × *g* for 5 min), washed twice, and resuspended in phosphate buffered saline (pH 7.2). The OD of the suspension was adjusted with phosphate buffered saline to 0.7 (±0.2) at 595 nm. Three microliter of bacterial cell suspensions was overlaid with 1 mL of *n*-nonane (Alfa Aesar, Lancashire, U.K.) as the hydrocarbon, and was added to 1.5 mL of 2 M ammonium sulfate (Kanto chemical, Tokyo, Japan). The suspensions were then vortexed for 3 min (Ab). A tube containing 3 mL of the untreated cell suspension was used as a control (Ac). All tubes were allowed to stand at room temperature for 30 min. Following incubation, 1 mL of the lower aqueous layer was removed using a pipette and the $OD_{600nm}$ was measured. The absorbance ratio of the bacterial assay tubes (Ab) relative to the control suspension (Ac) was calculated as a percentage of bound cells to the hydrocarbon using the following formula, (%) = (Ac − Ab)/Ac × 100.

## Colony spreading

TSB supplemented (0.24% or 0.75%) agar was autoclaved at 121 °C for 15 min. Sterile medium (20 mL) was poured into a petri dish (90-mm dia). The plates were dried for 20 min in a safety cabinet. Bacterial overnight culture (2 $\mu$L) was spotted onto

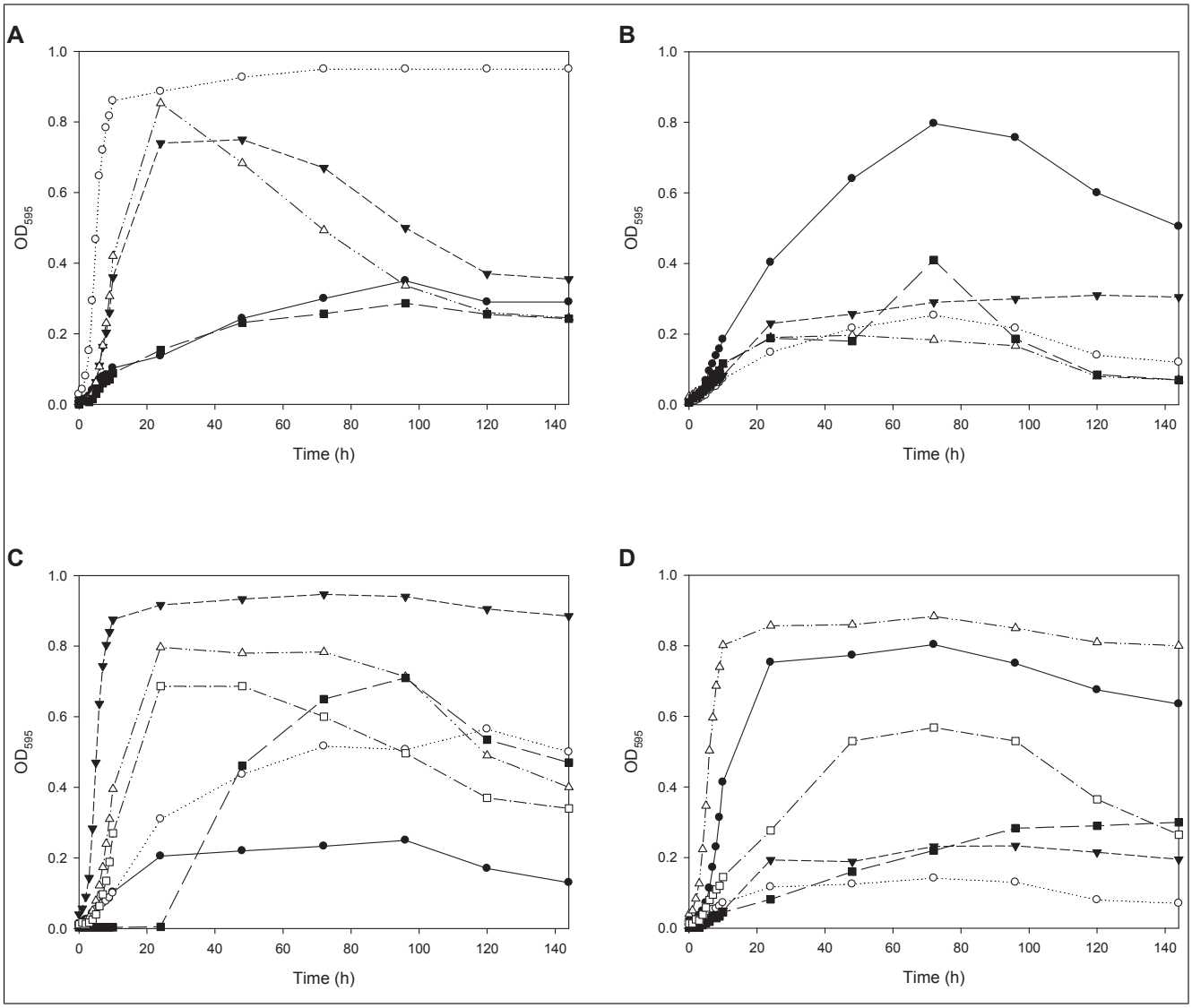



Figure 3–Growth of 22 spoilage bacteria isolated from fresh produce at 25 °C.
(A) ●, *Chryseobacterium balustinum*; ○, *Enterobacter* sp.; ▼, *Pantoea agglomerans*-1; △, *Pantoea agglomerans*-2; ■, *Bacillus pumilus*. (B) ●, *Clavibacter michiganensis*; ○, *Pseudomonas fluorescens*-1; ▼, *Pseudomonas fluorescens*-2; △, *Acinetobacter clacoaceticus* -1; ■, *Acinetobacter clacoaceticus*-2. (C) ●, *Stenotrophomonas maltophilia*-1; ○, *Stenotrophomonas maltophilia*-2; ▼, *Klebsiella pneumonia*; △, *Dickeya zeae*; ■, *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc2; □, *Pantoea agglomerans* (RDA-R). (D) ●, *Dickeya zeae* (RDA-R); ○, *Acinetobacter clacoaceticus* (RDA-R); ▼, *Stenotrophomonas maltophilia* (RDA-R); △, *Klebsiella pneumonia* (RDA-R); ■, *Bacillus pumilus* (RDA-R); □, *Clavibacter michiganensis* (RDA-R).

the center of the plates and dried for 10 min in a safety cabinet. The plates were covered and incubated at 37 °C for 10 h (Kaito and Sekimizu 2007).

### Statistical analysis

Data were analyzed by the analysis of variance (ANOVA) method using the SAS version 9.1 software package (Version 9.1 SAS Inst., Cary, N.C., U.S.A.) for the randomized analysis. If the results were significant ($P \leq 0.05$), the means were compared using Duncan's multiple range tests. Correlation coefficients were calculated by ANOVA.

## Results and Discussion

### Growth characteristics of spoilage bacteria

The growth of spoilage bacteria stored at 5 and 15 °C were examined over 1 mo, while those stored at 25 and 35 °C were evaluated over 3 d, and the resulting growth curves are shown in Figure 1 to 4. As the temperature increased, the growth of spoilage bacteria also increased. At 25 and 35 °C, the spoilage bacteria reached the stationary phase in less than 20 h on average. At 5 °C, on average more than 200 h were required to reach stationary phase and less than 100 h was required for 15 °C storage. However, the stationary phase spoilage bacteria in this study were incubated longer at 5 °C than the other 3 temperatures. Like most microorganisms, the spoilage bacteria in this study showed a relative inhibition of growth at 5 °C, especially *B. pumilus*, *D. zeae*, *A. clacoaceticus*-1, *D. zeae* (RDA-R), and *B. pumilus* (RDA-R), which showed almost no growth. However, the numbers of some spoilage microorganisms (*Enterobacter* sp., *P. agglomerans*-1, *P. agglomerans*-2, and *K. pneumonia*) multiplied at low temperature (Figure 1).

Table 1 presents the growth rate and lag time of spoilage bacteria at 5, 15, 25, and 35 °C using the modified Gompertz equation

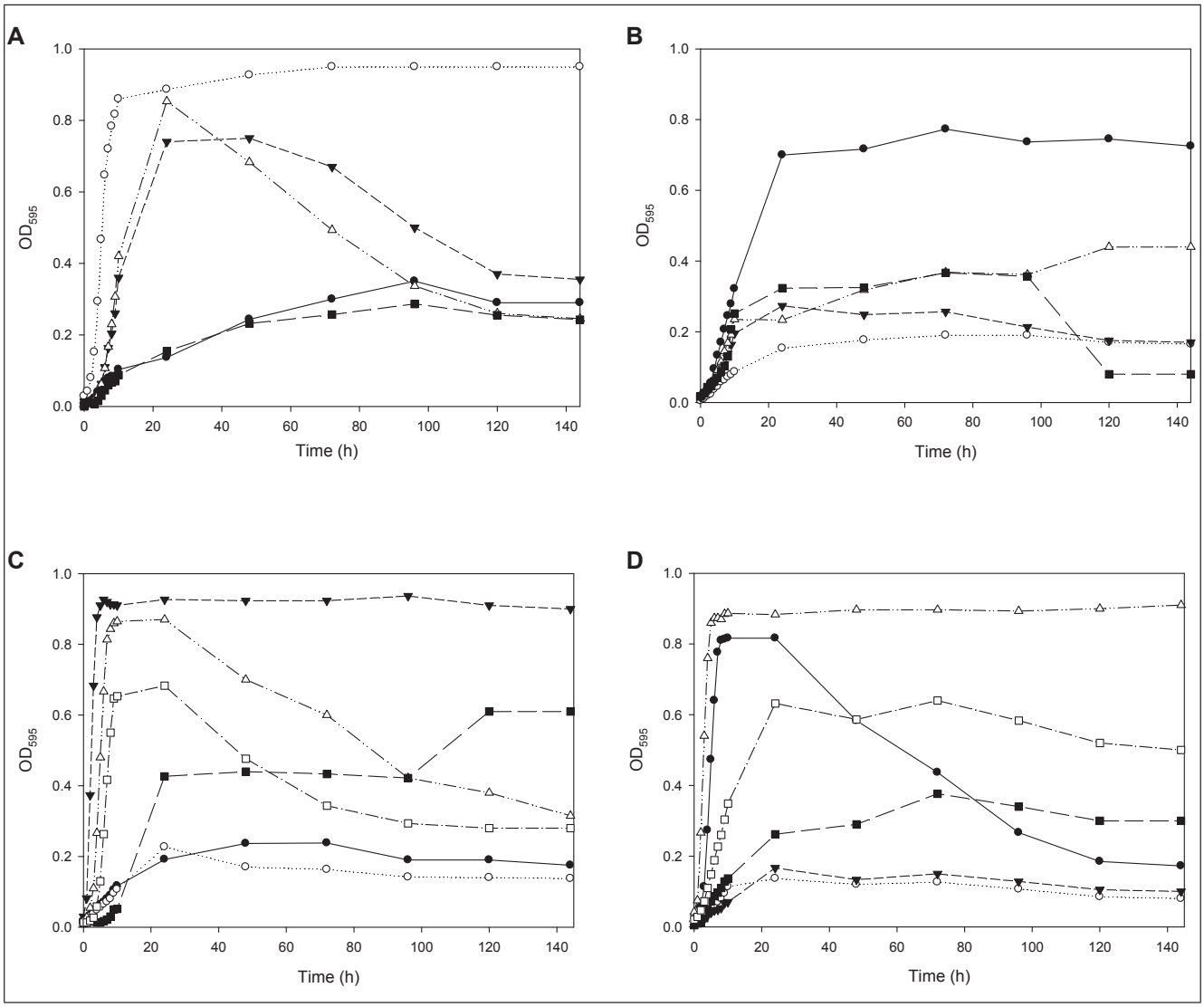


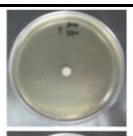
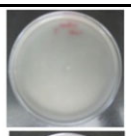
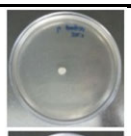
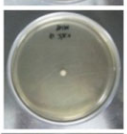
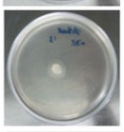
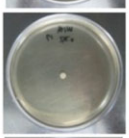
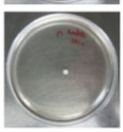
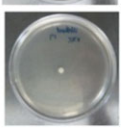
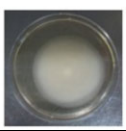
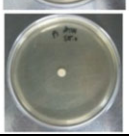
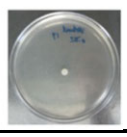

Figure 4–Growth of 22 spoilage bacteria isolated from fresh produce at 35 °C.
(A) ●, *Chryseobacterium balustinum*; ○, *Enterobacter* sp.; ▼, *Pantoea agglomerans*-1; △, *Pantoea agglomerans*-2; ■, *Bacillus pumilus*. (B) ●, *Clavibacter michiganensis*; ○, *Pseudomonas fluorescens*-1; ▼, *Pseudomonas fluorescens*-2; △, *Acinetobacter clacoaceticus*-1; ■, *Acinetobacter clacoaceticus*-2. (C) ●, *Stenotrophomonas maltophilia*-1; ○, *Stenotrophomonas maltophilia*-2; ▼, *Klebsiella pneumonia*; △, *Dickeya zeae*; ■, *Pectobacterium carotovorum* subsp. *Carotovorum* Pcc2; □, *Pantoea agglomerans* (RDA-R). (D) ●, *Dickeya zeae* (RDA-R); ○, *Acinetobacter clacoaceticus* (RDA-R); ▼, *Stenotrophomonas maltophilia* (RDA-R); △, *Klebsiella pneumonia* (RDA-R); ■, *Bacillus pumilus* (RDA-R); □, *Clavibacter michiganensis* (RDA-R).

**Table 1–Growth rate and lag time of 22 spoilage bacteria at 5, 15, 25, and 35 °C of 3 replicates.**

| Strains | Growth rate ($h^{-1}$) | | | | Lag time (h) | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 °C | 15 °C | 25 °C | 35 °C | 5 °C | 15 °C | 25 °C | 35 °C |
| *C. balustinum* | $0.45^{ABa}$ | $0.32^{B}$ | $0.45^{B}$ | $0.41^{B}$ | $75.36^{B}$ | $4.80^{EFG}$ | $0.00^{D}$ | $2.80^{B}$ |
| *Enterobacter* sp. | $0.53^{A}$ | $0.80^{AB}$ | $0.89^{B}$ | $0.81^{AB}$ | $46.13^{B}$ | $5.05^{EFG}$ | $2.62^{BCD}$ | $2.30^{B}$ |
| *P. agglomerans*-1 | $0.38^{ABC}$ | $0.64^{B}$ | $0.73^{B}$ | $0.75^{AB}$ | $52.27^{B}$ | $10.08^{CDEFG}$ | $6.17^{BCD}$ | $4.10^{B}$ |
| *P. agglomerans* -2 | $0.54^{A}$ | $0.69^{B}$ | $0.84^{B}$ | $0.65^{AB}$ | $91.58^{B}$ | $13.82^{CDEFG}$ | $6.25^{BCD}$ | $5.00^{B}$ |
| *B. pumilus* | NG[b] | $0.17^{B}$ | $0.34^{B}$ | $0.63^{AB}$ | NG | $12.85^{CDEFG}$ | $1.03^{BCD}$ | $0.83^{B}$ |
| *C. michiganensis* | $0.45^{AB}$ | $0.62^{B}$ | $0.82^{B}$ | $0.75^{AB}$ | $74.32^{B}$ | $19.74^{BCD}$ | $4.91^{BCD}$ | $4.77^{B}$ |
| *P. fluorescens* -1 | $0.26^{ABCDEF}$ | $0.25^{B}$ | $0.25^{B}$ | $0.23^{B}$ | $96.60^{B}$ | $29.49^{AB}$ | $5.04^{BCD}$ | $1.15^{B}$ |
| *P. fluorescens* -2 | $0.20^{ABCDEF}$ | $0.25^{B}$ | $0.28^{B}$ | $0.25^{B}$ | $171.83^{B}$ | $7.42^{DEFG}$ | $6.11^{BCD}$ | $3.65^{B}$ |
| *A. clacoaceticus* -1 | $0.01^{EF}$ | $1.65^{A}$ | $0.20^{B}$ | $0.36^{B}$ | $100.58^{B}$ | $0.75^{FG}$ | $3.19^{BCD}$ | $4.43^{B}$ |
| *A. clacoaceticus* -2 | $0.04^{CDEF}$ | $0.58^{B}$ | $0.08^{B}$ | $0.40^{B}$ | $63.47^{B}$ | $0.00^{G}$ | $8.10^{B}$ | $4.33^{B}$ |
| *S. maltophilia* -1 | $0.20^{ABCDEF}$ | $0.22^{B}$ | $1.09^{B}$ | $0.29^{B}$ | $95.70^{B}$ | $8.91^{CDEFG}$ | $3.19^{BCD}$ | $1.63^{B}$ |
| *S. maltophilia* -2 | $0.26^{ABCDEF}$ | $0.29^{B}$ | $0.51^{B}$ | $1.19^{AB}$ | $78.42^{B}$ | $14.34^{CDEF}$ | $5.73^{BCD}$ | $3.79^{B}$ |
| *K. pneumonia* | $0.33^{ABCDE}$ | $0.76^{AB}$ | $0.87^{B}$ | $0.89^{AB}$ | $78.36^{B}$ | $4.78^{EFG}$ | $2.71^{BCD}$ | $1.21^{B}$ |
| *D. zeae* | NG | $0.61^{B}$ | $0.81^{B}$ | $0.84^{AB}$ | NG | $17.31^{BCDE}$ | $5.52^{BCD}$ | $3.07^{B}$ |
| *P. carotovorum* subsp. *C.* Pcc21 | NG | $0.57^{B}$ | $0.70^{B}$ | $0.60^{AB}$ | NG | $29.07^{BC}$ | $23.26^{A}$ | $24.99^{A}$ |
| *P. agglomerans* (RDA-R)[c] | $0.26^{ABCDEF}$ | $0.59^{B}$ | $0.71^{B}$ | $0.68^{AB}$ | $114.25^{B}$ | $21.03^{BCD}$ | $7.36^{BC}$ | $4.81^{B}$ |
| *D. zeae* (RDA-R) | $0.01^{DEF}$ | $0.68^{B}$ | $0.80^{B}$ | $0.80^{AB}$ | NG | $16.35^{BCDE}$ | $5.63^{BCD}$ | $2.92^{B}$ |
| *A. clacoaceticus* (RDA-R) | $0.14^{F}$ | $0.15^{B}$ | $0.14^{B}$ | $0.12^{AB}$ | $371.62^{A}$ | $10.18^{CDEFG}$ | $2.56^{BCD}$ | $2.86^{B}$ |
| *S. maltophilia* (RDA-R) | $0.16^{CDEF}$ | $0.21^{B}$ | $0.22^{B}$ | $0.78^{AB}$ | $138.18^{B}$ | $37.54^{A}$ | $8.10^{B}$ | $4.57^{B}$ |
| *K. pneumonia* (RDA-R) | $0.1^{BCDEF}$ | $0.67^{B}$ | $0.80^{B}$ | $0.84^{AB}$ | $97.20^{B}$ | $4.43^{EFG}$ | $3.24^{BCD}$ | $1.40^{B}$ |
| *B. pumilus* (RDA-R) | NG | $0.23^{B}$ | $7.10^{A}$ | $0.40^{B}$ | NG | $7.08^{CDEFG}$ | $3.60^{BCD}$ | $1.56^{B}$ |
| *C. michiganensis* (RDA-R) | $0.35^{ABC}$ | $0.49^{B}$ | $0.68^{B}$ | $0.71^{AB}$ | $33.37^{B}$ | $17.93^{BCDE}$ | $3.69^{BCD}$ | $2.84^{B}$ |

[a]Means with the same letter within a column are not significantly different ($P > 0.05$).
[b]"NG" means no growth.
[c]RDA-R, rifampicin-resistant bacteria.

**Table 2–Digital photography of representative spoilage bacteria colonies on colony spreading (0.24% or 0.75% agar) with or without dextrose in TSB at 37 °C.**

| Strains | 0.24% with dextrose | 0.75% with dextrose | 0.24% without dextrose | 0.75% without dextrose |
|---|---|---|---|---|
| *Enterobacter* sp. |  |  |  |  |
| *P. fluorescens* -2 |  |  |  |  |
| *S. maltophilia* -2 |  |  |  |  |
| *D. zeae* |  |  |  |  |

models. Table 1 demonstrates a significant difference of growth rates between the tested bacteria at 5 °C, including *B. pumilus*, *D. zeae*, *P. carotovorum* subsp. *Carotovorum* Pcc21, and *B. pumilus* (RDA-R), which had no growth in 5 °C. However, at 15, 25, and 35 °C, no significant difference between the tested bacteria was observed. Lag time also showed a significant difference between bacteria at 15 °C only ($P < 0.05$). For the tested bacteria in this study, *P. agglomerans*-2, *A. clacoaceticus*-1, and *S. maltophilia*-2 demonstrated the largest growth at 5, 15, and 35 °C, respectively. Finally, *A. clacoaceticus*-1 and *A. clacoaceticus*-2 showed the shortest lag time at 15 °C. *A. clacoaceticus* (RDA-R) and *S. maltophilia* (RDA-R) showed the significant increased lag time at 5 and 15 °C, compared to no antibiotic resistant strains, respectively ($P < 0.05$). And, the lag time of *B. pumilus* (RDA-R) was significantly different between rifampicin-resistant and no antibiotic resistant strains at 25 °C ($P < 0.05$). However, except for the case mentioned above, the similar growth characteristics of rifampicin-resistant strains and no antibiotic resistant strains were resulted for the growth rate and lag time of spoilage bacteria.

The psychrotrophic bacterium is one of the factors influencing food storage at lower temperatures. *Listeria monocytogenes*, a kind of psychrotrophic bacterium, is able to grow to a few degrees below

0 °C and can cause large outbreaks (Fang and others 2013). Valero and others (2007) reported that psychrotrophic *B. cereus* isolated from fresh vegetable and American salads were able to grow at 8 °C (3%) and 10 °C (87.9%). Also, Allende and others (2004) reported that psychographic bacteria counts increased 8 $\log_{10}$CFU/g in lettuce after 7 d at 5 °C. The spoilage bacteria including *Klebsiella* and *Pseudomonas* can cause spoilage in frozen foods and these spoilage bacteria were isolated 6.7% and 6.6% at vegetable samples of total 50, respectively (Manani and others 2006). Growth and survival of spoilage bacteria at low temperature was a very important issue associated with extending product shelf life. In this study, *Enterobacter*, *Pantoea*, *Klebsiella*, and *Pseudomonas* were grown to a maximum cell population after 8 d at 5 °C. However, while there were no significant differences in growth between 25 and 35 °C, spoilage bacteria both reached the stationary phase in less than 24 h. This result demonstrates that when fresh produce is kept at room temperature for more than 1 d, native microorganisms may be already growing. Keeping food refrigerated is an effective way to prevent food spoilage or prolong the shelf life of fresh produce. However, because of psychrotrophic spoilage bacteria growth at low temperature, extending the shelf life of fresh products will require a sterilization process before storage. And further studies are needed to applied growth of spoilage bacteria on fresh vegetables surface.

## Biofilm formation and cell hydrophobicity

The attachment to the surface of fresh products may be considered as a 1st step in the microbial spoilage of fresh products



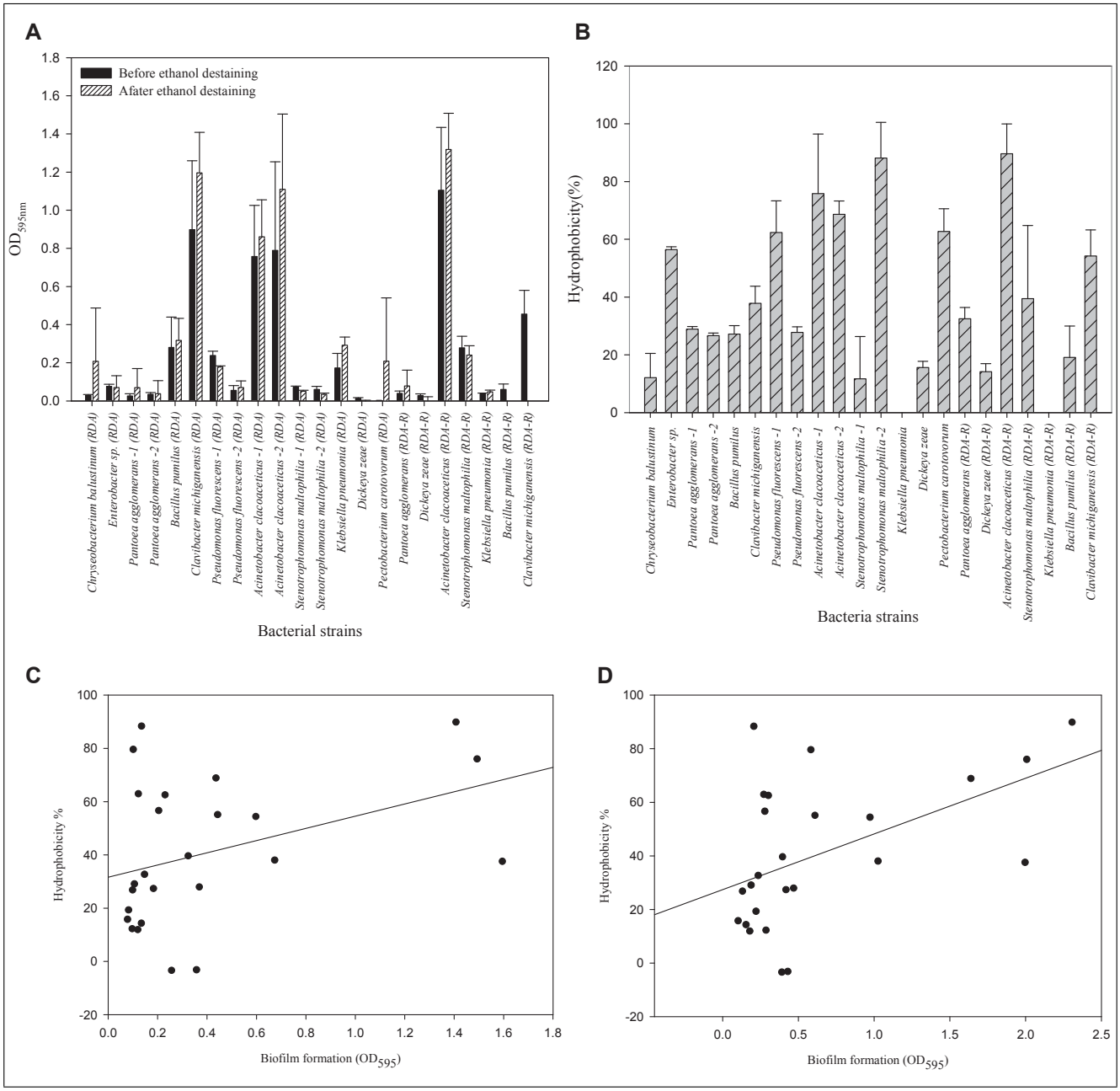



**Figure 5–Biofilm formation (A) and hydrophobicity (B) of spoilage bacterial strains isolated from fresh produce, and the correlation between biofilm formation of spoilage bacteria measured by cell hydrophobicity staining method with ethanol for 24 h, before ethanol (C, $r = 0.3762$) and after ethanol (D, $r = 0.4972$).**

(Benito and others 1997). Bacterial attachment to surfaces in and then biofilm formation influenced by physicochemical properties of environment (temperature and pH), cell hydrophobicity, and motility of microorganism (Herald and Zottola 1988; Choi and others 2013). Therefore, in this study, the ability of the spoilage bacteria to produce biofilms on 96-well polystyrene microtiter plates for 24 h measured by crystal violet staining is noted in Figure 5. Biofilm formation showed some differences among the test strains. The biofilm formation by *C. michiganensis*, *A. calcoaceticus*-1, -2, and *A. calcoaceticus* (RDA-R) ($OD_{595} > 0.6$) was greater than other bacteria. Conversely, *D. zeae*, *P. carotovorum* subsp., Carotovorum Pcc21, and *D. zeae* (RDA-R) showed nearly no biofilm formation ability. No significant differences before or after destaining using ethanol were observed ($P > 0.05$).

The result of cell hydrophobicity values of spoilage bacteria measured by the BATH method are shown in Figure 5B. High levels of cell hydrophobicity were observed in half of the strains tested. *Enterobacter* sp. (55.55%), *P. fluorescens*-1 (73.75%), *A. calcoaceticus*-1 (92.60%), *A. calcoaceticus*-2 (65.33%), *S. maltophilia*-2 (91.88%), *P. carotovorum* subsp., Carotovorum Pcc21 (53.89%), and *A. calcoaceticus* (RDA-R) (98.27%) showed a higher hydrophobicity (>50%) than others. Conversely, *C. balustinum* (19.34%), *S. maltophilia*-1 (13.00%), *K. pneumonia* (0.00%), *D. zeae* (RDA-R) (16.11%), and *K. pneumonia* (RDA-R) (0.00%) showed relatively low hydrophobicity levels (<20%) in the studied strains.

The correlation between biofilm formation measured by crystal violet staining, with or without ethanol decolorization and cell hydrophobicity are presented in Figure 5C and D. Biofilm formation in the strains incubated for 24 h was not highly correlated with the hydrophobicity level of the spoilage bacteria ($r = 0.3762$ and 0.4972) either before or after ethanol decolorization (Figure 5C and D).

Biofilm formation creates major problems in the food industry, because biofilms represent an important source of contamination, increased food spoilage (Choi and others 2013), and can support microbial growth (Donlan 2002; Teh and others 2010). In recent studies, biofilm formation of spoilage bacteria varied depending on bacterial strains. Liu and others (2013) reported that *K. pneumonia* strains exhibited strong biofilm formation ability on microtiter plates, and *P. fluorescens* strains displayed moderate biofilm formation. Besides, in this study, the biofilm formation of *K. pneumonia*, *P. fluorescens* showed moderate biofilm formation ability. The hydrophobicity of the bacterial cell surface may affect the surface attachment and biofilm formation of microorganisms. Benito and others (1997) reported a significant correlation between hydrophobicity and attachment strength to a meat surface of several pathogenic and spoilage bacteria. In our previous studies, several pathogens such as *Cronobacter sakazakii*, *Salmonella typhimurium*, *P. aerugenosa*, *L. monocytogenes*, and *Staphylococcus aureus* exhibited a high degree of correlation between hydrophobicity of the pathogenic cell and biofilm formation in strains incubated for 24 h (unpublished data). In this study, however, we observed a low correlation between hydrophobicity and biofilm formation of spoilage bacteria to fresh products. Auger and others (2009) similarly reported that hydrophobicity of *B. cereus* was not positively correlated with biofilm formation ($r = -0.23$). Cell surface hydrophobicity is reported to be one of the most important factors related to the mechanism of bacterial adhesion to inanimate biological surfaces (Vesterlund and others 2005; Lagha and others 2012). However, hydrophobicity values were not strongly correlated with the biofilm formation levels of the spoilage bacteria isolated from fresh products ($r = 0.3762$ to 0.4972). Biofilm formation and cell hydrophobicity of spoilage bacteria might vary depending on the spoilage bacteria strain. Therefore, further studies are needed to understand spoilage bacteria biofilm formation on surfaces.

### Biofilm formation and colony spreading

Colony spreading (0.24% soft agar) with and without dextrose showed a high spreading ability compared to colony spreading (0.75% soft agar) with and without dextrose. However, no difference was noted between soft agar samples with and without dextrose medium. *Enterobacter* sp., *B. pumilus*, *D. zeae*, *P. fluorescens*-2, *B. pumilus* (RDA-R), and *D. zeae* (RDA-R) demonstrated a high colony spreading ability compared to other strains (data not shown). Some representative spoilage bacteria colonies for colony spreading (0.24% or 0.75%) with or without dextrose are noted in Table 2. Colony spreading of the test spoilage bacteria in this study was stronger with 0.24% soft agar than with 0.75% soft agar. A negative correlation was observed between biofilm formation and colony spreading (0.24% or 0.75%), with and without dextrose ($r < 0.01$). Colony spreading (0.24% and 0.75% soft agar) and correlation range were 0.0057 to 0.0382 and −0.0074 to 0.0075, respectively (data not shown). It seems there is no relevance between biofilm formation and colony spreading, at least for the spoilage bacteria in this study. Many bacteria translocate by the propeller function of flagella (Kaito and Sekimizu 2007). However, Gram-negative bacteria such as *Escherichia coli* have the capacity to slide independent of flagella (Brown and Häse 2001). Furthermore, the colony spreading strains of *S. aureus* that do not have flagella, formed large amounts of biofilm (Kaito and Sekimizu 2007). Similarly in this study, spoilage bacteria isolated from fresh products have no correlation between colony spreading and biofilm formation.

## Conclusion

This study has shown the growth characteristics and biofilm formation ability of spoilage bacteria isolated from fresh products. There was no correlation between biofilm formation, cell hydrophobicity, or colony spreading. Growth at various temperatures and degrees of biofilm formation was different depending on the strains of spoilage bacteria. Low temperature storage can effectively delay the degeneration of fresh produce and extend the shelf-life of fresh produce. However, during transport and while the produce is in the store, the speed of degeneration will vary with the different temperatures. Understanding the factors that contribute to spoilage will allow us to target the main spoilage bacteria, which have rapid growth rates, to prolong freshness. Moreover, this characterization will provide a reference for forecasting the shelf-life of fresh produce at different storage temperatures.

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