Korean J. Food Sci. Ani. Resour.
Vol. 30, No. 6, pp. 946~950(2010)

ARTICLE

Behavior of *Burkholderia thailandensis* (*Burkholderia pseudomallei* surrogate) in Acidified Conditions by Organic Acids Used in Ready-to-Eat Meat Formulations under Different Water Activities

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Abstract

This study evaluated the antimicrobial effects of meat processing-related organic acids on *Burkholderia thailandensis* (*Burkholderia pseudomallei* surrogate) with different water activities. *B. thailandensis* KACC12027 (4 log CFU/mL) was inoculated in microwell plates containing tryptic soy broth pH-adjusted to 4, 5, 6, and 7 with ascorbic acid, citric acid, and lactic acid and with water activities adjusted to 0.94, 0.96, 0.98, and 1.0 with NaCl, followed by incubation at 35°C for 30 h. The optical density (OD) of the samples was measured at 0, 3, 6, 12, 24, and 30 h at 595 nm to estimate the growth of *B. thailandensis*. Growth of *B. thailandensis* was observed only at water activity of 1.0. In general, more bacterial growth ($p<0.05$) was observed at pH 6 than at pH 7, and the antimicrobial effects of the organic acids on *B. thailandensis* were in the following order: Ascorbic acid > lactic acid > citric acid after incubation at 35°C for 30 h. These results indicate that organic acids in meat processing-related formulations should be useful in decreasing the risk related to an emerging high risk agent (*B. pseudomallei*).

Key words: *Burkholderia thailandensis*, *Burkholderia pseudomallei*, dissociation constant

Introduction

Burkholderia pseudomallei is a Gram-negative bacterium which is usually isolated from soil, and the pathogen has been known to be related to melioidosis with significant morbidity and mortality up to 90% (Eoin West *et al*., 2008). This bacterium has recently brought attentions in food supply environments because *B. pseudomallei* shows broad host range and has caused disease in meat producing animals such as cattle, goats, and pigs (Sprague and Neubauer, 2004). In addition, animal-to-human transmission has also been reported, and most cases resulted in fatalities (Choy *et al*., 2000; Idris *et al*., 1998). Thus, the food contamination with *B. pseudomallei* is now considered as unavoidable (Dance, 2000). Li *et al*. (1994) also suggested that the meat from animals infected with *B. pseudomallei* would endanger the public health, and Zinchenko *et al*. (2008) recently conducted a study of *B. pseudomallei* on meat. In fact, there were melioidosis outbreaks caused by the ingestion of contaminated particles (Bassetti *et al*., 2005), drinking water (Inglis *et al*., 1998) and human breast milk (Ralph *et al*., 2004), and the recent risk assessment by Fosse *et al*. (2008) characterized *B. pseudomallei* as a bacterial hazard in European pork slaughter houses.

The number of infection cases with *B. pseudomallei* increase after flooding, and monsoon rainfalls are believed to be responsible for spreading *B. pseudomallei*, which leads to be a more severe infection in humans (Currie and Jacups, 2003; Ketterer *et al*., 1975). Taken together, it may suggest the possible ingestion of *B. pseudomallei* through animal origin foods such as pork and beef, especially for the region that has monsoon rainfalls. Therefore, the fate of *B. pseudomallei* needs to be studied under meat processing-related conditions. However, *B. pseudomallei* can be studied only in Biosafety Level (BSL)-3 facilities in many countries due to its high mortality rate (Bossi *et al*., 2004). *Burkholderia thailandensis* is genetically and morphologically very similar to *B. pseudomallei*, but it is not pathogenic to human (Qazi *et al*., 2008; Wuthiekanun *et al*., 1996), and *B. thailandensis* has also similar environment distribution with *B. pseudomallei* (Wuthiekanun *et al*., 1996; Yu *et al*., 2006). The

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differences between two species are only variations of arabinose-assimilation operon and the production of capsular polysaccharide (Haraga *et al*., 2008; Reckseidler *et al*., 2001). Hence, *B. thailandensis* has been used in recent studies related to physiological activities and pathogenic characteristics as a surrogate bacterium of *B. pseudomallei* (Haraga *et al*., 2008; Qazi *et al*., 2008; Thongdee *et al*., 2008; West *et al*., 2008; Yoon *et al*., 2010).

A variety of antimicrobials have been used to improve the food safety of manufactured foods (Feng *et al*., 2010). Most ready-to-eat meat products may contain various antimicrobials such as sodium chloride, lactic acid, nitrite and lactate/diacetate salts to inhibit *Listeria monocytogenes* growth (Glass *et al*., 1989; Mbandi and Shelef, 2002). In addition, the decrease of product pHs with organic acids such as ascorbic acid, citric acid, lactic acid, and acetic acid has been suggested in many studies to control pathogens. The previous studies by Calicioglu *et al*. (2002) and Yoon *et al*. (2005) showed that the treatments containing ascorbic acid, followed by heating at 60°C increased destruction of *Escherichia coli* O157:H7 in beef jerky slices. Recent studies also showed that the obvious antimicrobial effects of ascorbic acid and citric acid with chemical tenderizers on *E. coli* O157:H7 cells during marination at 4°C and cooking (Mukherjee *et al*., 2008, 2009; Yoon *et al*., 2009).

This study evaluated the antimicrobial activity of meat processing-related organic acids on emerging food-related agent (*B. pseudomallei*) using a surrogate (*B. thailandensis*) under different water activities.

Materials and Methods

Preparation of inoculum

B. thailandensis KACC 12027 (Korean Agricultural Culture Collection, Korea) stored as a frozen culture at -70°C was cultured in 10 mL of tryptic soy broth (TSB; Difco, Becton Dickinson and Company, USA) at 35°C for 24 h. The 0.1 mL of the culture was then transferred into 10 mL of TSB followed by subculture at 35°C for 24 h. Stationary phase cells were serially diluted with a saline solution to obtain an inoculum size of 4 log CFU/mL.

Treatments

To prepare various organic solutions with different levels of pH and water activities, the pH of TSB was adjusted to 4, 5, 6, and 7 with ascorbic acid (Sigma-Aldrich Corp., USA), citric acid (Duksan Pure Chemical Co., Ltd., Korea) and lactic acid (Junsei Chemical Co., Ltd., Japan), respectively, and the water activity for each pH level of the solutions was adjusted to 0.94, 0.96, 0.98, and 1.0 with NaCl (Duksan Pure Chemical). This resulted in a total of 16 combinations in a complete factorial design for each organic acid solution.

Inoculation and measurement of optical density

Each combination of organic acid solution was placed in wells of 96 micro-plate, and *B. thailandensis* was inoculated in each well to obtain 2 log CFU/mL. The micro-plates were then incubated at 35°C for 30 h. The OD (optical density) values of the samples were repeatedly measured at 0, 3, 6, 12, 24, and 30 h at 595 nm with a micro-plate reader (Bio-Tek®, ELx 808, Korea).

Statistical analysis

This experiment was repeated twice with two samples in each repeat. The OD values in interactions among the fixed factors such as organic acid solutions, pH, water activity and incubation time were analyzed by the mixed model procedure of SAS® version 9.2 (SAS Institute, Cary, USA). The type III F-test was used to remove random effects in the model at alpha=0.05 by the forward stepwise method, and the interaction effects of fixed effects and random effects were also examined in the type III F-test. Multiple mean comparisons among the interaction (organic acid pH × water activity × incubation time) were performed with the pairwise *t*-test at alpha=0.05.

Results and Discussion

Bacterial growth of *B. thailandensis* was observed only 1.0 of water activity of solutions (F-value=864.0; $p<0.0001$) (Figs. 1-3). Thus, data are presented and discussed only for organic acids which had 1.0 of water activity in the text. Although bacterial growth was observed in some samples treated with organic acids, the growth appeared after 12 h of lag phase duration, regardless of the type of organic acid (Figs. 1-3).

In the conditions treated with ascorbic acid, only the samples adjusted to pH 6 and 7 with the organic acid showed dramatic bacterial growth ($p<0.05$) of *B. thailandensis* after lag phase duration, but not below pH 5 (Fig. 1B). However, OD_{595} values of *B. thailandensis* was significantly higher ($p<0.05$) in pH 6 of TSB broth (0.625±0.078) than in TSB (0.391±10.02) with pH 7 after incubation at 35°C for 30 h (Fig. 1B). The similar result was also observed under the conditions of TSB treated with lactic acid, and higher ($p<0.05$) OD_{595} value (0.156±0.022) was observed

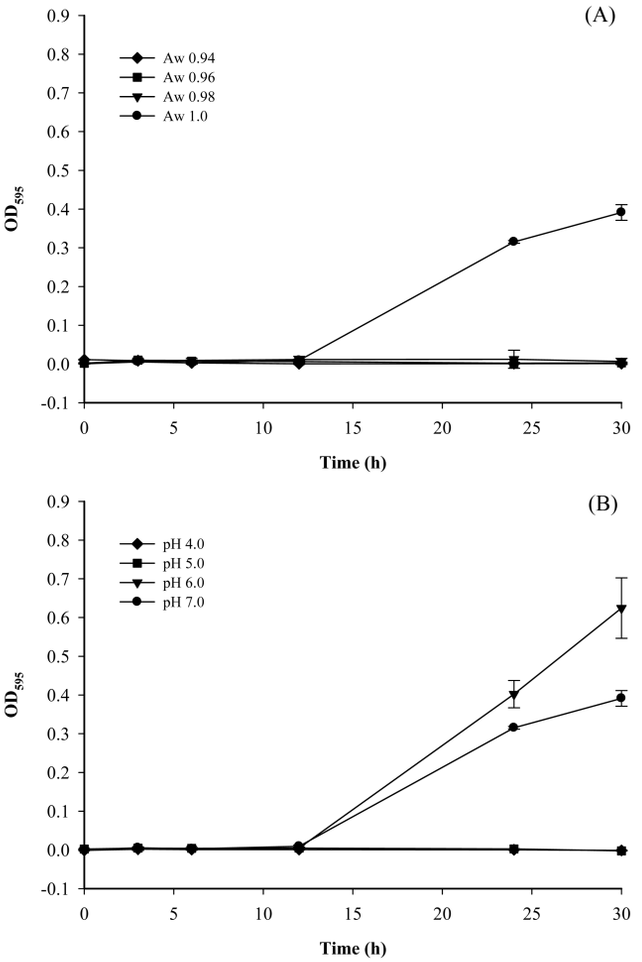

Fig. 1. Optical density (595 nm) of *Burkholderia thailandensis* KACC12027 under different water activities at pH 7.0 of ascorbic acid (A) and under different acidified conditions by ascorbic acid at 1.0 of water activity (Aw) (B) during incubation at 35°C for 30 h.

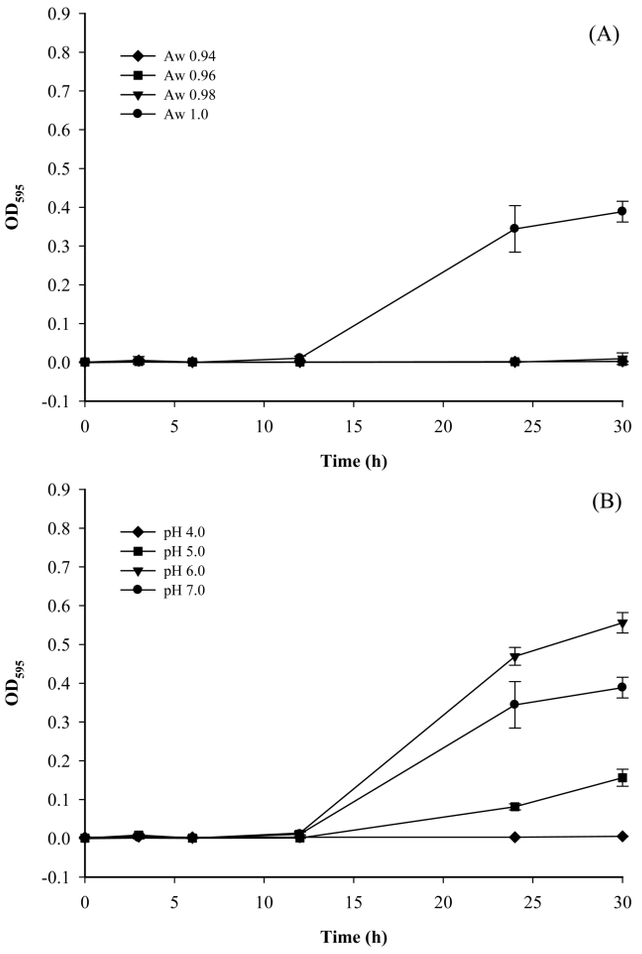

Fig. 2. Optical density (595 nm) of *Burkholderia thailandensis* KACC12027 under different water activities at pH 7.0 of lactic acid (A) and under different acidified conditions by lactic acid at 1.0 of water activity (Aw) (B) during incubation at 35°C for 30 h.

in the samples (pH 5) adjusted with lactic acid than the one (-0.003±0.001) adjusted with ascorbic acid to pH 5 after incubation at 35°C for 30 h (Figs. 1B and 2B). These results indicate that *B. thailandensis* can have more growth in acidic condition than in neutral condition, which was not observed in *L. monocytogenes* under the similar conditions (Yoon and Choi, 2010). This could be related to the pathogenic mechanisms of *B. thailandensis*. The bacteria uses type III secretion system to invade host, but type III secreted proteins such as BipD and BopE were detected only under acidic condition. Moreover, *B. thailandensis* under acidified environment (pH 4.5) induced the capability of the bacterium to invade human respiratory epithelial cells A549 (Jitprasutwit *et al*., 2010). The result showing more growth in pH 6 than pH 7 became more obvious in citric acid-treated samples (Fig. 3B). Interestingly, the samples treated with the organic acid to pH 5 (OD_{595}=0.753±0.083) had higher (p<0.05) optical density, compared to the samples having higher pH values (Fig. 3B). The order of OD_{595} for the samples treated with citric acid was as follow; pH 5 (0.753±0.083) > pH 6 (0.582±0.006) > pH 7 (0.386±0.009) at 35°C for 30 h (Fig. 3B).

Although the pH values of samples treated with different organic acids were at the same pH values, antimicrobial effects of the organic acids on *B. thailandensis* were ascorbic acid > lactic acid > citric acid (F-value=107.86; p<0.0001) (Figs. 1-3). This order was opposite to the order of the dissociation constants (citric acid: 7.510^{-4} > lactic acid: 1.3810^{-4} > ascorbic acid: 6.7610^{-5}). Organic acids with lower dissociation constants have higher undissociated molecules. The undissociated molecules of organic acid permeate through the microbial cell membrane and dissociate into charged ions in the cytosol

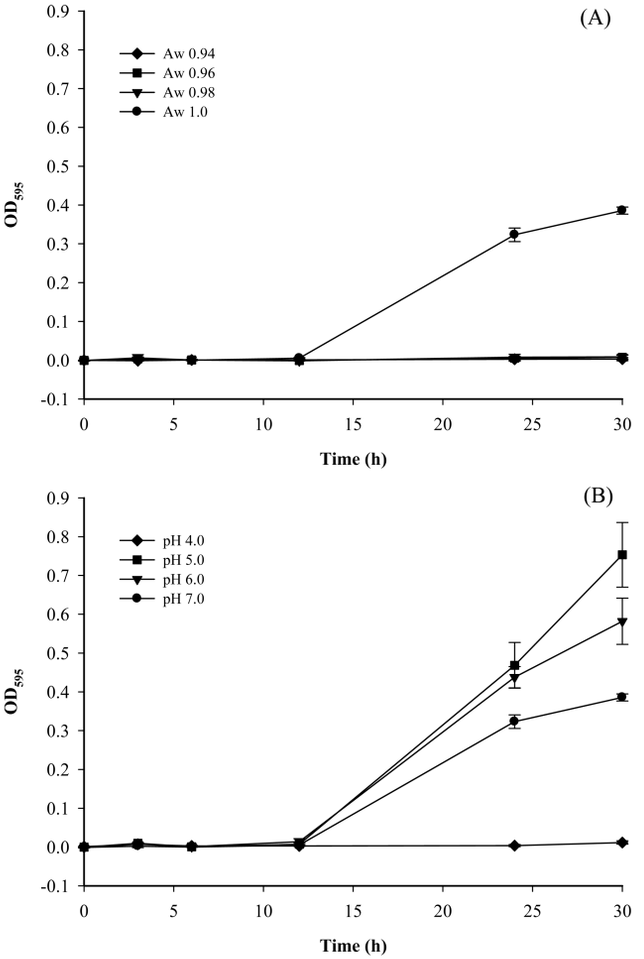

Fig. 3. Optical density (595 nm) of *Burkholderia thailandensis* KACC12027 under different water activities at pH 7.0 (A) of citric acid and under different acidified conditions by citric acid at 1.0 of water activity (Aw) (B) during incubation at 35°C for 30 h.

resulting in the cells to deplete energy reserves to pump out the ions across the plasma membrane (Booth and Kroll, 1989; Bracey *et al*., 1998). It is well known that the antimicrobial activity of organic acids is mainly associated with their undissociated state (Ahamad and Marth, 1989). Therefore, ascorbic acid showed more inhibition on the growth of *B. thailandensis* than lactic acid followed by citric acid.

In conclusion, use of organic acid in meat processing-related formulation should be useful in decreasing the risk related to a emerging high risk agent (*B. pseudomallei*) in food-related conditions, but level of acidification should be determined according to the dissociation constant of organic acid because some organic acid such as citric acid allowed even more growth of *B. thailandensis* at pH 5.

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(Received 2010.11.3/Revised 2010.12.3/Accepted 2010.12.6)