Plant diseases caused by soil-borne pathogenic fungi, such as *Fusarium* and *Pythium*, affect vegetables and crops growth and yield worldwide, and even result in significant economic loss (Noble and Coventry, 2005; Asad et al., 2014). A synthetic chemical fungicide has long been used for reducing the incidence of soil-borne plant diseases. However, it causes soil, water and air pollution, and is harmful to the health of people (Pieters and Vlietinck, 2005). With increasing attention to environmental protection and the increasing market demand for organic products, biological control exhibits excellent performance (Brannen and Kenney, 1997; Zheng et al., 2013). Some antagonistic microorganisms, such as *Bacillus*, *Pseudomonadaceae*, *Agrobacterium* and *Pasteurella*, can produce antimicrobial compounds which have been proved to be quite effective in controlling various plant diseases (Shrestha et al., 2014). Among them, *Bacillus subtilis* is one of the most effective microbes in controlling plant diseases (Nagórska et al., 2007). It has been reported that *B. subtilis* can not only inhibit the growth of pathogenic fungi by producing various lytic enzymes and antibiotics, but also induce systemic plant resistance by increasing the activities of several enzymes related to plant defense (Baysal et al., 2008; Choudhary and Johri, 2009; Santoyo et al., 2012). Furthermore, it is easy to cultivate and stabilize in the environment by forming spores (Lalloo et al., 2009). These characteristics provide *B. subtilis* with potential advantages over the other antagonistic microorganisms in the production and preservation of biocontrol agents (Chen et al., 2010b; Nagórska et al., 2007). It must be recognized that the efficacy of biocontrol agent prepared with antagonistic bacteria largely depends on the count of viable bacteria. One of the persistent challenges is assuring acceptable stability in the development of a biocontrol agent. The allowable level of loss of viable bacteria in biocontrol agent should be generally less than 10%.

In our previous work, a biocontrol bacterium, *B. subtilis* B579, which exhibited obvious growth inhibition effect against pathogens, *Rhizoctonia solani*, *Fusarium gramineum*, *Fusarium solani*, *Fusarium oxysporum* and *Phytophthora capsici*, was obtained (Chen et al., 2010a; Chen et al., 2013). In the present study, the relation of viable bacteria to the storage period and temperature of biocontrol agent of *B. subtilis* B579 was calculated using the accelerated aging method.

**Expiration Date Prediction of Biocontrol Agent Prepared with *Bacillus subtilis* B579 Using the Accelerated Aging Method**

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**Abstract**

The expiration date of biopesticidal products is an essential feature of their use and storage. In the present work, the expiration date of biocontrol agent was predicted using the accelerated aging method. The available bacteria in *Bacillus subtilis* B579 biocontrol agent were 3.7 ± 0.2 × 10¹¹ CFU/g. It is calculated that the expiration date of the agent was about 17 months at 25°C. During this period, the available bacteria retained more than 90% of the value in the initial product. Thus, this work suggests the expiration date of biocontrol agents composed with spores could be estimated using the accelerated aging method.

**Key words**: *Bacillus subtilis*, accelerated aging, biocontrol agent, expiration date
The pathogen was incubated in the centre of PDA plates at 28°C for 2 days. Then, 200 μl of fermentation broth, biocontrol agent, negative solution (agent without B. subtilis B579) or sterile deionized water was added into the oxford cup. The concentrations of biocontrol agent and negative solution were 1 g/l. The fermentation broth was diluted to 3.7 × 10^{11} CFU/l which was the same as that of biocontrol agent solution. After 2 days incubation at 28°C, their inhibition effects were compared.

The antagonism of biocontrol agent (1 g/l) against F. oxysporum was determined on PDA plates using the dual plate assay method (Tang et al., 2010). Sterile deionized water served as a control. Fungal radial growth was measured with vernier calliper. The fungitoxicity was recorded in terms of percentage growth inhibition which was determined as [(Dc – Dt)/Dc] × 100%. Where Dc was the average increased diameter of the fungal colony in control, and Dt was the average increased diameter of the fungal colony in treatment (Amini et al., 2010). Three replications were carried out for each treatment.

The expiration date of the biocontrol agent of B. subtilis B579 was predicted by the accelerated aging method (Waterman and Adami, 2005). The expiration date was defined as the time when the survival cell number of the agent was 90% of the initial cell number.

The inactivity of B. subtilis B579 cells follows the first order kinetics process. Under certain temperature, the equation can be represented as the equation (1).

\[
\log N_t = \log N_0 - k_c \cdot t / 2.303
\]  

\[
(1)
\]

\(N_0\) is the initial cell concentration of agent, and \(N_t\) is the concentration of survived cells at time \(t\). \(k_c\) is the rate constant under the temperature \(T\). The accelerated test was conducted at temperatures of 50°C, 60°C, 70°C, 80°C and 90°C. At each temperature, the survived cell number was detected at 0 day, 1 day, 2 day, 3 day, 4 day, 5 day, and 6 day, respectively. The \(k_c\) at each temperature could be obtained based on the linear regression between \(\log \ N_0\) and \(\log \ t\).

Arrhenius exponential law is the theory evidence of accelerated aging test, of which the logarithmic form can be expressed as equation (2).

\[
\log k_c = -\frac{E_a}{2.303 R} \cdot \frac{1}{T} + \log A
\]  

\[
(2)
\]

Where, \(k_c\) is the rate constant calculated from equation (1) at temperature \(T\), \(E_a\) stands the activity energy, and \(R\) is gas constant. A regression equation could be obtained based on the linear relation between \(\log k_c\) and \(1/T\). For example, the \(k_{579}\), the rate constant at 25°C, could be calculated from \(\log k_{579}\). Thus, the period for 10% cell inactivity of the agent at 25°C could be calculated according to the equation (1), namely, the expiration date of the biocontrol agent.

As shown in Fig. 1, the inhibition zones of biocontrol agent, fermentation broth, negative solution and sterile deionized water were 10.0 ± 0.7 mm, 11.3 ± 0.6 mm, 0.5 ± 0.1 mm and 0 mm (no inhibition), respectively. The negative solution which was used for agent preparation showed little inhibitory effect on F. oxysporum. The inhibition zone of biocontrol agent was a little less than that of fermentation broth because some lytic enzymes such as chitinase and β-1, 3-glucanase were produced during the fermentation (Chen et al., 2010a). This result indicated that the B579 biocontrol agent could effectively inhibit the growth of plant pathogen F. oxysporum. The inhibition effect of 72.4 ± 4.2% against F. oxysporum was obtained at 1 g/l of the biocontrol agent.

Temperature is one of important factors determining the survival of B. subtilis B579 in the agent. In this study, temperatures were set as 50°C, 60°C, 70°C, 80°C and 90°C, separately, to investigate the expiration date of biocontrol agent using the accelerated aging method. The number of survival cells in the agent at
variable temperatures was detected, and the results are listed in Table I. The inactivity rate of cells in the agent increased with the increase of temperature. The regression equation under different temperature conditions was obtained as shown in Fig. 2. The rate constant at temperature 25°C, $K_{25}$, was calculated as $2.042 \times 10^{-4}$ d$^{-1}$. Therefore, $t_{0.9}$, i.e. the time when the number of surviving cells was 90% of the initial agent, was calculated as 514 d according to the equation (1). The expiration date of the $B. subtilis$ B579 biocontrol agent was predicted as 17 months at 25°C.

The accelerated aging method is usually used for predicting the expiration date of drugs in a relatively short time (Waterman and Adami, 2005). To our knowledge, few studies have been reported on the expiration date prediction of biocontrol agent prepared with antagonistic bacterial cells. Commonly, the inactivity of bacteria cells is different from that of chemical drugs because most bacteria are sensitive to heat. However, spores of $B. subtilis$ are heat resistant, and are in a state of metabolic dormancy (Setlow, 1994). Luckily, the inactivity of agent containing $B. subtilis$ B579 spores follows the first order kinetics process. The $R^2$ of regression equation under 50°C, 60°C, 70°C, 80°C and 90°C was 0.9811, 0.9913, 0.9847, 0.9768 and 0.9733, respectively. Thus, the inactivity kinetics of biocontrol agent prepared with spores could be analyzed using Arrhenius equation. The present work suggests that the expiration date of biocontrol agent prepared with spores of antagonistic bacteria could be predicted with the accelerated aging method which is effective and timesaving.

Nevertheless, additional work is required, since the inhibition effect of fermentation broth against $F. oxysporum$ was a little more than that of biocontrol agent with the same cell number. Besides chitinase and $\beta$-1,3-glucanase, some antibiotics (Iturin A and Surfactin) that were thermal stable were identified from fermentation broth of $B. subtilis$ B579 by the method of HPLC-MS (data not shown). Therefore, a more efficient biocontrol agent, containing antibiotics that are produced during the fermentation, might be obtained by spray drying of fermentation broth.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (days)</th>
<th>Logarithm of relative cell concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>323 K (50°C)</td>
<td>0</td>
<td>2.000</td>
</tr>
<tr>
<td>333 K (60°C)</td>
<td>0</td>
<td>2.000</td>
</tr>
<tr>
<td>343 K (70°C)</td>
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<td>2.000</td>
</tr>
<tr>
<td>353 K (80°C)</td>
<td>0</td>
<td>2.000</td>
</tr>
<tr>
<td>363 K (90°C)</td>
<td>0</td>
<td>2.000</td>
</tr>
</tbody>
</table>
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Literature


