JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. 7(4), p. 3043-3050

Inactivation of *Bacillus subtilis* by a Pulsed Magnetic Field and Kinetics Model

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(Received: 23 September 2013; accepted: 06 November 2013)

The pulsed magnetic field (PMF) is a non-thermal method for inactivating microorganisms at lower temperatures and preserving the food sensory and nutritional qualities. In the present study, inactivation performances of *Bacillus subtilis* with the various pulsed magnetic field (PMF) intensities and pulses were well investigated. Results showed that the *B. subtilis* survival rate generally decreased with the increasing intensity (>1.5T) and pulse numbers (>15) and a minimum survival rate of 33.87% was obtained at an intensity of 3.3 T and pulse number of 30. However, lower intensity (≤1.5T) and PMF pulse numbers (≤15) enhanced the *B.subtilis* growth. By comparing the determination coefficients (r^2), bias factors (*Bf*), accuracy factors (*Af*) and root mean square errors (*RMSE)*, the Weibull distribution model with the highest r^2 and the lowest *RMSE* was selected as the optimal kinetic model to describe the PMF inactivation process. Scanning Electron Microscope (SEM) observations indicated that PMF inactivated the *B. subtilis* cells by deformating the cell surface and damaging the cellular membrane. These findings for the first time present the kinetic model and the cell death mechanism during the PMF inactivation process, and will provide a useful reference for further PMF sterilization application.

Key words: Pulsed magnetic field (PMF); *Bacillus subtilis*; Inactivation; Kinetics model; SEM.

Many processing methods have been currently used in the food industry to produce safe products and preserve food with high quality. Thermal processing is a typical method to increase shelf life and maintain food safety with low processing costs[1]. However, an increasing trend toward to produce more fresh-like foods improves the possibility of replacing thermal treatments with other alternative processing methods. Non-thermal methods can inactivate microorganisms at lower temperatures with a minimal loss of fresh quality, nutritional value and color/flavor and now have been received a wide interest[2]. Some examples of non-thermal processes include high hydrostatic pressure (HHP), pulsed electric fields (PEF), high-intensity ultrasound (US), and oscillating magnetic fields[3].

The pulsed magnetic field (PMF) is a non-thermal process for inactivating microorganisms. For example, Hofman presented an effective oscillating magnetic field inactivation for microorganisms in food[4]. Li also found that PMF was able to inactivate *Escherichia coli* cells at B=160 mT with t=16 h and a pulse frequency of *f*=62 kHz, leading to a considerable destruction level of $N/N_0 = 10^{-4}$ for the *E. coli* cells[5]. However, some results of PMF inactivation efficiency are

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controversial. For example, San Martin showed that PMF treatment had no significant effect on microbial populations[6]. A pulsed magnetic field of 18 T was regarded as being incapable of causing *Escherichia coli* inactivation, even when it was combined with other technologies (that is, HHP, PEF, US, or anti-microbial agents). Some data indicated that these controversial results might be partially attributed to "window effect".

Our research group has focused on the inactivation of microorganisms in milk[7], foremilk[8], watermelon juice[9], and beer[10] by using pulsed magnetic field since 2003. Several microorganisms including *E. coli*, *S. aureus*, and *S. cerevisiae* have been used for testing PMF inactivation efficiency[11]. The previously obtained results showed that PMF had the inactivation effect on microorganisms in the liquid culture. The species of microorganisms, PMF parameters including magnetic field intensity and pulse number, environmental factors including the ionic concentration, temperature, and pH, and physiological factors including microbial cell concentration and population growth phase affected significantly the PMF inactivation efficacy. The inactivation inconsistency induced by the magnetic field intensity and pulse number was an interesting phenomenon, which was also described by the window characteristics of the non-thermal biological effects associated with magnetic fields[11]. Therefore, to determine the effect of PMF intensity and pulse number on inactivation of microorganisms is necessary. And the related mechanism of PMF inactivation on microorganism has never been investigated up to now.

Bacillus subtilis is an important spoiler microorganism in the food industry with the potential of causing economic losses and/or health problems[12]. Several non-thermal treatments including infrared irradiation, high pressure, and supercritical CO_2 have been tested to inactivate *Bacillus subtilis*[13-15]. However, few studies of PMF treatment with *B. subtilis* can be found in the literature. During the inactivation process, kinetic models are necessary to be developed for defining the processing conditions of PMF and finally achieving a certain microbial safety level. The Bigelow model was the first one to transfer the bacteriological and physical data to the thermal calculation of canned food processes[16] and subsequently to demonstrate the logarithmic nature of death time curves[17]. This concept has served the canning industry well. The original equation was recently modified to model the combined effect of three variables on microorganism inactivation[18,19]. The Weibull equation has also been adapted to nonlinear survival curves and has been applied to several microorganisms[20,21] while Hülsheger model was the first one to describe the survival curves based on a relationship between the logarithm of the microbial survival fraction and the treatment time at specific electrical field intensity[22].

The objectives of the present study were: 1) to investigate the inactivation efficiency of PMF treatment against *B. subtilis* with the various intensity and pulse number levels, 2) to select a optimal model among the Bigelow, Weibull distribution function and the Hülsheger models for describing the inactivation process, and 3) to investigate the primary mechanism of the PMF treatment on the *B. subtilis* by the changes of cell morphological diversity.

MATERIALS AND METHODS

Microorganism culture

B. subtilis strain ATCC 6633 was obtained from the American Type Culture Collection (ATCC) and was used for this study.

A stock culture of *B. subtilis* ATCC 6633 was prepared by inoculating the activated cells in a slant containing 5 g of peptone, 3 g of beef paste, 5 g of NaCl, 0.05 g of $MgSO_4$, 15 g of agar, and 1000 mL of distillted water with a final pH of 7.0. The activated cells were obtained by transferring the stock culture into 50 mL of liquid medium with the same composition as the stock media, and the cells were incubated at 30°C for 12 h. Next, 5 mL of the activated bacterial liquid was inoculated into 50 mL of fresh liquid medium and incubated at 30°C or 10 hours (Cells were in log phase). Finally, 5 mL of these bacterial cultures were transferred into plastic tubes and subjected to PMF treatment.

PMF treatment

A bench-scale continuous unit manufactured in Jiangsu University (China, P.R.) was used to treat *B. subtilis* vegetative cells. The PMF was supplied by a well regulated DC power supply and generated by automatically alternating the charge and discharge to the chamber coil using

a series of capacitances. The heating device and cooling system were connected within the treatment chamber to maintain the selected temperature at 25 °C. The PMF intensity in the treatment chamber was recorded by a Tesla meter. The pulse number was manually counted. The PMF system generated a maximum magnetic field intensity of 3.3 Tesla (T).

For selecting the optimal operational parameters, a magnetic field intensity from 1.0 T to 3.3 T and pulse numbers from 5 to 30 were applied to the bacterial culture at room temperature (25 ± 1°C). Each experiment was repeated at least three times.

Bacterial counts were conducted by diluting and counting the resulting colony-forming units (CFU).

The inactivation efficiency was evaluated by calculating the survival rate q (%) of the bacteria with the following equation (1):

$$q(\%) = \frac{N}{N_0} \times 100\% \quad ...(1)$$

where N and N_0 represent the total bacterial counts after and before inactivation, respectively.

Inactivation models

To establish the appropriate treatment conditions and to achieve known levels of microbial inactivation, a reliable model that accurately described the survival rate of *B. subtilis* by PMF was needed. The actual experimental data were applied to three kinetic inactivation models for a fitness comparison. The mathematical models are as follows:

Bigelow (1921)[19]

$$\log(S) = -\frac{t}{D} \quad ...(2)$$

where S is the survival rate q (%)/100 at treatment time t (represented by pulse number), and D is the decimal reduction time, or mathematically speaking, the negative inverse of the inactivation curve slope.

Weibull (1951) distribution function[23]

$$\ln(S) = -(\frac{t}{a})^b \quad ...(3)$$

where a and b are the scale and shape factors, respectively; the b factor interprets the shape of the survival curve such that when $b < 1$, the survival curve is concave (it forms tails), $b>1$ indicates that the survival curve is convex (forms shoulders) and $b = 1$ indicates a straight line on the ln-scale (equal to the Bigelow model).

Hülsheger et al. (1981)[22]

$$\ln(S) = -b_t \ln(\frac{t}{t_c}) \quad ...(4)$$

where b_t is the regression coefficient, t_c is the most critical treatment time (the longest treatment time in which the survival rate equals 100%).

Statistical analysis

The experimental data were fitted to the models by nonlinear regression procedures in SPSS software (SPSS Institute Inc., Chicago, IL, USA). The analyses of variance were assigned a confidence level of p=0.05 and additional regression analyses were performed to establish relationships between the estimated parameters and operational conditions.

The models were tested and their accuracy was assessed by determination coefficients (r^2), bias factors (*Bf*), accuracy factors (*Af*) and root mean square errors (*RMSE*)[24]. The measured survival rate values were plotted against the estimated values, with the r^2 values indicating the fitting accuracy. The higher the r^2 value, the more suitable the model was for describing the data. The *Bf* is a bias factor that indicates a systematic over- or underestimation of growth. The *Af* indicates by how much the estimated data differ from the measured data. The *RMSE* measured the average deviation between the measured and estimated values. The equations (5, 6, and 7) that were used to calculate *Bf*, *Af* and *RMSE* are as follows:

$$Bf = 10^{\frac{\sum \log(SP/SM)}{n}} \quad ...(5)$$

$$Af = 10^{\frac{\sum |\log(SP/SM)|}{n}} \quad ...(6)$$

$$RMSE = \sqrt{\frac{\sum (SM-SP)^2}{n-1}} \quad ...(7)$$

Where n is the number of measurements,

SP is the estimated survival rate, and *SM* is the experimental survival rate.

Scanning Electron Microscopy (SEM)

The untreated and PMF-treated suspensions of *B. subtilis* cells were centrifuged at 3000 rpm for 10 min. The suspensions were later washed two times with phosphate buffer (PB, 0.1 M, pH 7.4). Cells were then resuspended in 1 mL phosphate buffer. The suspensions were fixed in glutaraldehyde and post-fixed with 1% osmium tetraoxide. After fixation, the cells were sequentially dehydrated with 50, 70, 80, 90, 95% and 100% ethanol for 15 min each. Samples were then coated with gold-palladium. Samples was observed under a scanning electron microscope (Hitachi S4800, Japan) operating at 15 kV accelerating voltage, and photomicrographs were obtained.

RESULTS AND DISCUSSION

Effect of PMF intensity and pulse number on inactivation efficiency

The effect of PMF intensity and pulse number on inactivation efficiency is shown in Fig. 1. The survival rate of *B. subtilis* generally decreased with an increase in PMF intensity from 2 T to 3.3 T and the survival rates of all trials reached a minimum at a PMF pulse number of 30. At 3.3 T and 30 pulse numbers, the survival rates of *B. subtilis* reached a minimum level of 33.87%. However, the number of bacterial cells increased at 1.0 T and 1.5 T at pulses of less than 15. Survival rates were especially high with 10 pulses compared with 5 and 15 pulses at 1 T and 1.5 T of intensity. The phenomenon that PMF improved growth of *B. subtilis* may attribute to the combied effect of the low intensity and low pulse number. A pulse number of 10 at 1.0 T and1.5 T was probably the "time window" that improved *B. subtilis* growth maximumly. A similar phenomenon was also determined in microorganisms that were treated with an electromagnetic field of low intensity and frequency. For example, a pulsed electromagnetic field with an amplitude of 0.5 mT and 50 Hz resulted in a proliferation rate of over 25% in yeast suspensions[25].

Window effect characteristics of the electromagnetic field was revealed when samples were treated with electromagnetic waves at extremely low frequency and low intensity[26]. The "window effect" indicates that targets inside biological systems only respond to electromagnetic waves at a discrete frequency or intensity range, known as the "frequency window" or "intensity (or power density) window", above and below the range of which frequency or intensity display no biological effect on organisms. The "time window" was identified by Byus and Tauscher[27]. According to the PMF that was generated by a DC power supply, the window effect can occur with respect

Table 1. Kinetics constants of the Bigelow Model

Intensity/T	*D*	r^2	*Bf*	*Af*	*RMSE*
2.5	95.81±20.20	0.836	0.918	1.098	0.094
3	74.21±11.10	0.909	0.950	1.071	0.064
3.3	68.72±3.32	0.994	1.070	1.070	0.044

r^2, determination coefficient; *Bf*, bias factor; *Af*, accuracy factor; *RMSE*, root mean square error. [a]Values±confidence in the interval at p=0.05

Table 2. Kinetics constants of the Weibull distribution function

Intensity/T	*b*	*a*	r^2	*Bf*	*Af*	*RMSE*
2.5	1.78±0.38	36.33±3.10	0.915	1.017	1.028	0.058
3	1.31±0.22	31.80±2.03	0.937	1.012	1.060	0.053
3.3	0.88±0.054	27.96±0.71	0.994	1.000	1.022	0.013

r^2, determination coefficient; *Bf*, bias factor; *Af*, accuracy factor; *RMSE*, root mean square error. [a] Values±confidence in the interval at p=0.05

Table 3. Kinetics constants of the Hülsheger Model

Intensity/T	b_t	t_c	r^2	*Bf*	*Af*	*RMSE*
2.5	0.30±0.11	4.91±2.20	0.661	1.000	1.117	0.097
3	0.40±0.11	4.35±1.69	0.754	1.000	1.134	0.091
3.3	0.46±0.06	3.61±0.73	0.935	1.000	1.065	0.046

r^2, determination coefficient; *Bf*, bias factor; *Af*, accuracy factor; *RMSE*, root mean square error.
[a] Values±confidence in the interval at p=0.05

to intensity and pulse number (exposure time). Perhaps "window effect" can provide a good explain for the research results that PMF had no additional inactivation effect or cell damage on *E. coli*. 50 pulses or 18 T appeared outside the range of "time window" at 18 T or "intensity window" at 50 pulses that PMF can inactivate *E. coli*.

When treated with PMF pulses higher than 15, all survival rates decreased. At 30 pulses, the survival rates decreased from 79.36% to 33.87% with a corresponding increase of intensity from 1 T to 3.3 T.

Alternating the magnetic field with an intensity higher than 2.0 T is regarded as a high-frequency magnetic field and has a stronger inactivation effect[28]. When the PMF intensity was higher than 2.0 T, the bacterial survival rates decreased with an increase in the pulse number. At 3.3 T, the survival rates decreased from 66.67% to 33.87% with the increase in the pulse number from

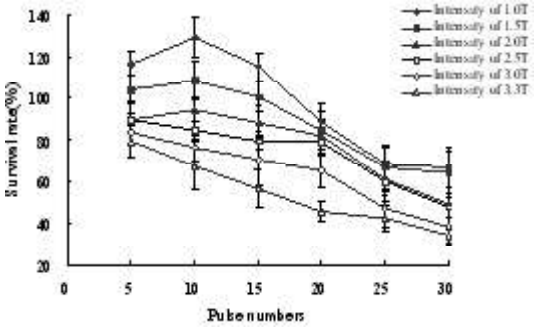

Fig. 1. Magnetic field intensity and pulse number effects on the survival rate of *B. subtilis*

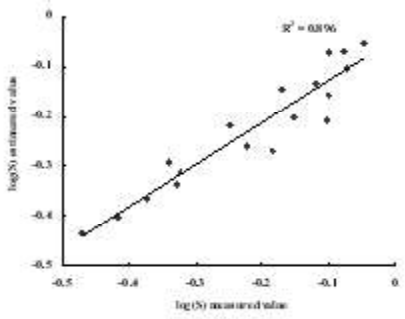

Fig. 2. Plot of measured values vs. estimated values for the Bigelow model

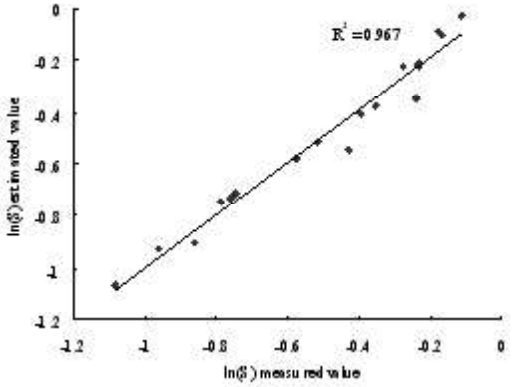

Fig. 3. Plot of measured values vs. estimated values for the Weibull distribution function

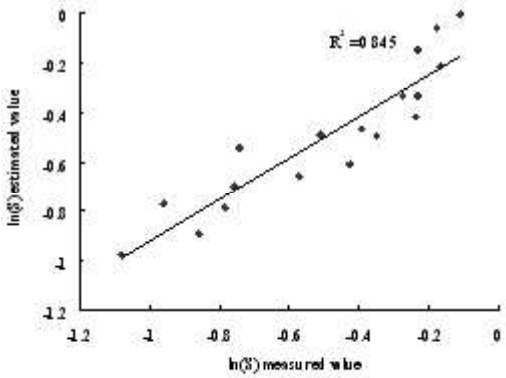

Fig. 4. Plot of measured values vs. estimated values for the Hülsheger model

5 to 30. Therefore, a higher PMF intensity and pulse number were essential for *B. subtilis* inactivation.

Inactivation kinetic model of *B. subtilis* in response to PMF treatment

The survival curves of *B. subtilis* cells that were exposed to PMF had a characteristic sigmoid shape when plotted in linear coordinates, as shown in Fig. 1. Bigelow, Weibull and Hülsheger models were used for the experimental data when the PMF intensity was over 2.0 T.

Tables 1–3 show the parameter values that were calculated for the Bigelow and Weibull distribution function and the Hülsheger models. In Table 1, the Bigelow model gave relatively high determination coefficients (0.836-0.994). The *Bfs* (0.918-1.070) were within the proposed acceptable limits, i.e., 0.75-1.25[29], indicating a good predictive performance according to the proposed limits. The *Af* was also within the acceptable upper limit (1.070-1.098), accounting for the finding that *Af* typically increases by 0.1-0.15 for every variable in the model[30]. The *D* values decreased from 95.81 to 68.72 and along with the increase of PMF intensity, indicating that PMF intensity played an important role in *B. subtilis* inactivation.Table 2 presents the parameters (scale factor, *a*; shape factor, *b*) and regression parameters for the Weibull distribution function model. The Weibull distribution function fits well with the experimental data (r^2, 0.915) and exhibited good accuracy (*Af* =1.022-1.060). The *Bf* of the model was above 1, indicating that this model slightly overestimated the PMF effect by approximately 1.2% and 1.7% with intensities of 2.5 T and 3.0 T, respectively. The *RMSE*s were at low levels from 0.013-0.058. The *b* values decreased from 1.78 to 0.88 and the *a* values decreased from 36.33 to 27.96 with an increase in magnetic intensity from 2.5 T to 3.3 T. Table 3 shows the parameters that were calculated for the Hülsheger model. This model yielded low r^2-values (0.661-0.935) and high *RMSE* (0.046-0.097). The *Afs* were far from 1 (1.065-1.134). Therefore, the Hülsheger model could not describe the PMF inactivation process of *B. subtilis* with acceptable accuracy.

The *Af* and *Bf* values of the Bigelow model were near 1, but the model was less reliable compared to the Weibull distribution function because the Bigelow model had lower values for the r^2 and higher values for the *RMSE*. The Weibull distribution function could do well in predicting the inactivation process of *B. subtilis* with PMF treatments, given that it had the highest r^2 values,

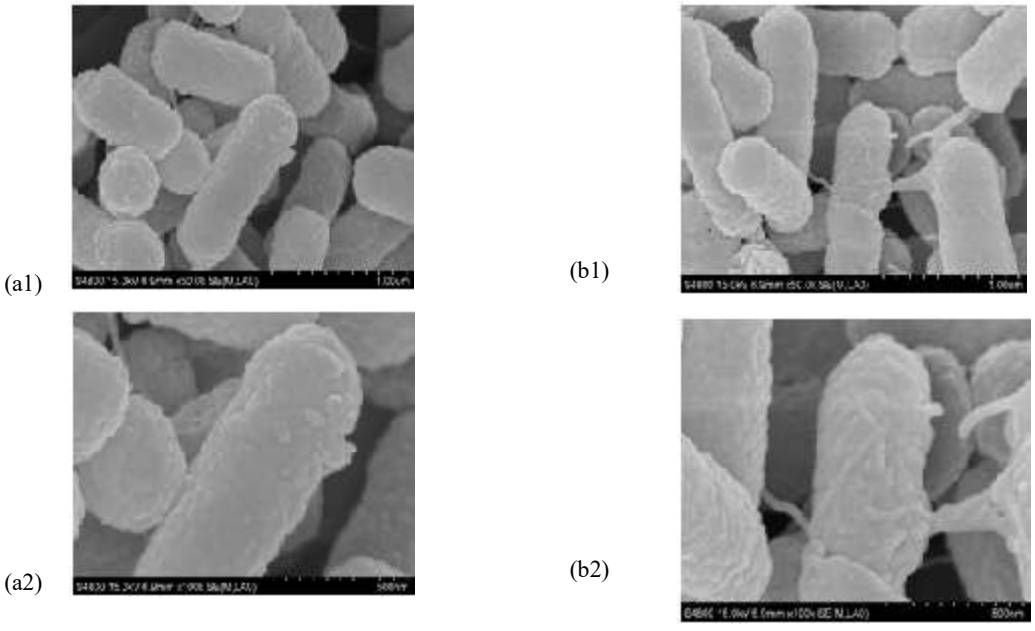

Fig. 5. SEM micrographs of *B. subtilis* before and after PMF treatment (a1, b1: 50000× magnification; a2, b2: 100000× magnification). (a1),(a2): images of PMF-untreated cells; (b1),(b2): images of PMF-treated cells at an intensity of 3.3 T and 30 pulses

the best predictive *Bf,* a stable *Af* (nearest to 1) and the lowest *RMSE* values. The Hülsheger model was not a candidate for describing the *B. subtilis* inactivation because the Hülsheger model produced lower r^2-values, a higher *RMSE*, and an *Af* that was far from 1 compared with the Bigelow and Weibull distribution function models.

The actual measured and estimated values are shown for the Weibull model in Fig. 3. The correlation R^2 value of 0.967 for the linear regression analyses between measured and estimated values also showed that the predicted Weibull frequency distribution function fit well with the measured data, while the Bigelow and Hülsheger models had lower R^2 values between the measured and estimated values (Fig. 2, 4).

Morphology changes of PMF-treated *B. subtilis* cells

Effects of PMF on the *B. subtilis* morphology have been investigated. Scanning electron microscopy (SEM) showed visible changes in their morphology (Fig. 5). The untreated cells were intact and has smooth surface. But the cells treated by PMF with intensity of 3.3T and 30 pulsed numbers showed wrinkles and deformations on their surface. The treated cells were not broken into pieces but showed discontinuities in the membrane compared to untreated cells, which suggested that cell membrane was physically damaged by PMF.

Cell death involving cell membrane has generally been attributed to mechanical disruption of the cell wall or membrane. Cell membrane controls the cell's metabolic activities by maintaining an osmotic balance between the cell and its surrounding environment. Any damage to the cell membrane could break this balance and lead to the cell death. In this study, PMF might inactivate *B. subtilis* by causing cellular membrane disruption, which further led to the loss of cytosol and DNA from cells, and finally caused the cell death of *B. subtilis*. Further research are needed to verify these possible mechanisms.

CONCLUSION

A bench-scale continuous PMF unit was set up for the inactivation treatment of *B. subtilis* in this study. Our investigation showed that the PMF intensity and pulse number were key factors in the PMF inactivation. Low pulse numbers and relatively low intensity benefited the growth of *B. subtilis*. A higher PMF intensity and pulse number were generally required for *B. subtilis* inactivation. The maximum inactivation ratio of 33.87% was obtained with 3.3 T and 30 pulses. Several models were applied to describe PMF inactivation. Compared with the models from Bigelow and Hülsheger, the Weibull frequency distribution function had the best fit with the experimental data, which had the highest r^2, lowest *RMSE*, lowest *Bf* and *Af* values that were closest to 1. This model could elucidate the inactivation process characteristics of *B. subtilis* and be a useful reference for further developing and optimizing the inactivation processes in combination with other inactivation technology. SEM observation was consistent with those of inactivation and also confirmed the *B. subtilis* cell death, which indicated that PMF possibly inactivate the *B. subtilis* by the disruption of the cellular membrane.

The effectiveness of PMF treatments depended on the processing parameters, including the intensity and pulse numbers. Further research is needed to inactivate *B. subtilis* spores by PMF with higher intensities and pulse numbers or using PMF in combination with other inactivation technology.

ACKNOWLEDGMENTS

The authors wish to thank the National Natural Science Foundation of China (Grant No.31271966) and Research and Innovation Project for Postgraduate of Higher Education Institutions of Jiangsu Province (Grant No.CX10B-021X) for supporting this project.

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