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Effect of nano-silver coating on microbial control of microwave-freeze combined dried sea cucumber**

Xinlin Li[1] Min Zhang[1] Xu Duan[1], and Arun S. Mujumdar[2]*

[1]State Key Laboratory of Food Science and Technology, Jiangnan University, 214122 Wuxi, Jiangsu, China
[2]Department of Mechanical Engineering and Engineering Science Program, National University of Singapore, 9 Engineering Drive 1, Singapore 117576

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A b s t r a c t. To develop a new microbial control method for microwave-freeze dried sea cucumber, nano-silver coating is tested as a potential technique. According to the experimental results, a 0.3 mg l^{-1} nano-silver coating can control over 99% of *Bacillus subtilis* in the process of microwave-freeze drying. To ensure a total microbial control effect, the nano-silver coating technique was combined with the microwave-freeze drying process. Nano-silver coating treatment was found to be effective in lowering the microorganism count and had no significant effect on drying efficiency.

K e y w o r d s: nano-silver coating, sea cucumber, microbial control, microwave-freeze drying

INTRODUCTION

Sea cucumbers (*Stichopus japonicus*) belong to the *Holothurioidea* of *phyum Echinodermata* and are extremely rich in nutrients and tonic function, containing acid mucopolysaccharide, sea cucumber saponin, and other bioactive substances (Cui *et al.*, 2007). Santiago-Cardona found that the sea cucumber lipopolysaccharide (LPS) can be used as a catalyst for immunisation, through the immune regulation that can significantly increase the expression of protease SAA (Santiago-Cardona, 2003). Rajesh Kumar isolated from sea cucumber triterpene saponin from *Actinopyga lecanora.* The saponin showed in vitro antifungal activity against twenty fungal species, and was found to be most effective against *Trychophyton mentagrophytes* and *Sporothrix schenckii*, at MIC range of 1.56 μg ml^{-1} (Kumar, 2007). *S. japanicus* is the most popular edible sea cucumber in China (Liao, 2001). Aside from the high nutritional value of sea cucumber, its transportation and storage are very difficult, because sea cucumbers can autolyse after leaving sea water within a few hours. It can lose 80% in long-distance transportation and it is very difficult for the inland people to eat fresh sea cucumbers. As a result, most sea cucumbers in the market are dehydrated products, processed with various drying methods. The traditional method of processing is salt drying, and the main component of the body wall of sea cucumber is collagen (Li and Fu, 2004). Vacuum freeze drying can maintain the original shape of the material, and also preserve the rich nutrition and bioactive substances of sea cucumbers; it is considered as the best among current processing methods.

Compared with other drying methods, vacuum freeze drying is the best drying method of sea cucumber (Yun and Han, 2006), but the drying process needs at least 18 h and the cost is very high. The temperatures are relatively low in the whole process of freeze drying, and the microorganisms are difficult to kill, so most of the microorganisms become dormant but survive. In the next place, the freeze drying product easily absorbs moisture because of the existence of a highly developed network structure which provides good conditions for microbial growth and reproduction. To obtain good quality product with lower cost, microwave-freeze drying (MFD) was used for sea cucumber drying.

The method is called microwave-freeze drying (MFD) as microwaves are used as the heat source for freeze drying (Zhang *et al*., 2007), and it is more convenient and efficient than other hybrid drying methods. Few studies have been conducted on the application of MFD for food materials. For example. The MFD was used to dry some liquid material,

*Corresponding author's e-mail: min@jiangnan.edu.cn
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such as aqueous pharmaceutical excipient, aqueous mannitol solution and skimmed milk (Wang and Chen, 2003, 2005; Wang *et al*., 2005). What is more, sterilization can be conducted during the MFD process, due to the rapid heating rate and non-thermal effect of microwaves (Bell and Myrick, 2001; Dibrov, 2002; Duan *et al*., 2007). It would be a good idea to combine microwave technology and freeze drying as it provides lower costs and sterilization.

However, it is not enough to use microwaves to control microorganisms, because the nutritional components of sea cucumber are destroyed if the temperature is too high. Besides, another problem of microwave heating is non-uniform temperature distribution in treated materials, and there are some cold spots on the materials, which could allow microorganism survival. So it was essential to find a new technology to control microorganisms while obtaining good quality product.

To ensure the health safety of products, nano-silver coating was adopted for the control of microorganisms. It appears to be a good choice to apply nano-silver coating before microwave-freeze drying of sea cucumbers, because nano-silver is a kind of broad-spectrum antimicrobial material with long-term, non-resistant, non-toxic side effects, and other advantages (Dibrov and Dzioba, 2002). Nano-silver has a broad-spectrum antibacterial properties because silver particles can easily penetrate into the cells of organisms and inhibit the activity of some enzymes (Bell and Myrick, 2001; Dibrov, 2002; Blahovec, 2007). Up to now, it has been used as an antibacterial agent in many fields, such as medical treatment, textile and dope *etc*. (Ah *et al.*, 2004; Lee and Jeong, 2005; Rhim and Hong, 2006; Mitrus and Mościcki, 2009; Yin and Zhang, 2007). Nano-silver can be used as food preservative film and packaging material with antimicrobial activity. An *et al*. (2008) studied green asparagus nano-silver coating preservation. Nano-silver, applied as a coating on sea cucumber, could compensate the disadvantages of microwave sterilization, if the dose of addition could be controlled within a safe range.

In the study presented in this paper, MFD technology was used to dry sea cucumbers, with the introduction of nano-scale silver coating pretreatment.

The aim of this study was to investigate the effects of nano-silver coating pretreatment on the drying process of sea cucumber and on microbial control of *Bacillus subtilis*, and on storage characteristics of MFD products.

MATERIALS AND METHODS

Sea cucumbers, *Stichopus japonicus*, were purchased and transported from Shanghai, China. The lab scale dryer used in this study, in which the freeze drying (FD) and MFD operations can be carried out simultaneously, was developed by the authors. The dryer has two drying chambers where FD and MFD can be carried out, respectively. When materials are dried in the FD cavity, they are heated by an electric heating plate. If samples are dried in the MFD chamber, microwaves supply the heat. During the drying process, required pressure is maintained by a vacuum pump, and the temperature of the cold trap is low enough to condense vapour. In order to avoid non-uniform distribution of the microwave field, three magnetrons are used, positioned at different angles. The microwave power can be adjusted continuously. The temperature of materials is detected by optic fibre which can work well in the microwave field.

Other main instruments, equipments and chemicals used in the experiment were as follows: a steam sterilizer (ZDX-35BI, Shanghai Medical Nuclear Instruments Factory, China), an air clean bench (SW-CJ-10, Su Zhou Sanxing Air Clean Technology Co, Ltd., China), an electro-heating standing-temperature cultivator (SPX-80B, Shanghai Yuejin Medical Instruments Factory, China), a cabinet laboratory dryer (101-1BS, Shanghai Medical Appliance Factory, China), a nano-particle size analyzer (Nano-ZS 90, Malvern Instruments Ltd., England), a microwave digester (MARS5, CEM company, USA), and an atomic absorption spectrometer (220Z, Varian Comp., USA).

$AgNO_3$ with 1.7 g was dissolved in 20 ml of deionized water to form Ag^+ solution, and warmed up in 47°C water bath. 1.2 g NaH_2PO_2 H_2O,0.25 g sodium hexametaphosphate and 2.7 g PVP were mixed and dissolved in 125 ml of deionized water, then put into 500 ml conical flask and 2.5 ml H_2SO_4 was added (c = 1.0 mol l^{-1}) to form the deoxidizer. Ag^+ solution was dripped (25~35 droplets/min) into deoxidizer which was put on constant temperature magnetic stirring apparatus keeping 47°C. After the dripping operation and stirring for 60~90 min the nano-scale silver solution was obtained.

Oxidized starch with 7.5 g weight was dissolved in 150 ml of deionized water, then 1.8 g glycerol was added, and the whole was warmed up in 70°C water bath and stirred till completely dissolved. Then the temperature was reduced to 40°C, 0.6 mg l^{-1} of nano-silver was added to the 150 ml, stirred for 20 min. After the completion of this procedure, the solution was subjected to 30 min ultrasonic treatment at the power of 0.5 W ml^{-1}.

In order to comply with the hygienic standard for dried aquatic products of animal origin (GB10144-2005), the nano-silver starch solution was diluted to 0.3 mg l^{-1}. In the experiments, the prepared nano-silver solution and nano-silver starch solution were used as coating agents.

In order to investigate the sterilization effects of the MFD process, the sea cucumber samples were not boiled so that microorganisms in the samples could remain until the drying process. After removing the gut and washing clean, sea cucumbers were drained for a while, and free water on the surface was removed with filter paper. Then, the whole body wall was immerged in the nano-silver solution for 1 min to form a coating film, followed by freezing at -25°C for at least 8 h.

In order to investigate the microbial control characteristics with nano-silver of different concentrations, while assessing the effect of nano-scale silver on the drying process,

experiments were carried out for MFD with different concentration coatings (MFD combined with nano-silver coating, MFD combined with nano-silver starch coating, MFD without nano-scale silver coating) at the same final moisture content of materials (6% w.b.). A batch of frozen sea cucumber body walls was dried at 4.2 W g^{-1} microwave power.

In order to assess the impact of MFD and nano-silver coating on different bacterial strains, experiments were conducted on inoculating *Bacillus subtilis* spores on the sterilised sea cucumber, then coating with nano-silver and drying with MFD. The power of microwave was 4.2 W g^{-1}.

All the drying processes as described above were carried out at 100 Pa chamber pressure and -40°C cold trap temperature. All the experiments were repeated three times and the average of the results was used for analyzes.

In order to study the changes of microbial number in coated sea cucumber in high humidity environment, the coated sea cucumbers were placed in a sample bottle, then weighed and placed within the scope of Comvita plate chamber. In the pre-room of the Comvita plate, saturated KNO_3 solution was added to the sample bottle, the bottle was sealed and placed in an incubator at 30°C. The total number of bacteria was assayed every five days, and changes of microbial populations of freeze dried sea cucumber in this film and humid environment were observed for a period of one month.

Granularity of nano-silver starch solution was determined by a nano-particle size analyser, based on dispersion or diffraction of monochromatic light. The particle size could be shown by different scattering angle, and by this measurement the granularity distribution of the whole sample could be determined.

Moisture content was determined by the oven method. At regular time intervals during the drying processes samples were taken and dried in the oven for 7-8 h at 105°C until constant weight. Weighing was performed on a digital balance, and then moisture content (w.b.) was calculated. The tests were performed in triplicate.

The dried samples were soaked in 25°C distilled water for 8 h, every 2 h, taken out and put on the filter paper of a Büchner funnel which was positioned on a suction flask evacuated for 30 s to remove free water from the sample surface. The sample weighing was performed in triplicate:

$$RR = \frac{W_r}{W_d} \qquad (\text{kg kg}^{-1}), \qquad (1)$$

where: RR - rehydration rate, W_r and W_d are masses of samples after and before rehydration.

Stored Program Control (SPC) can reflect the effect of sterilization, and its detection method is described by a Chinese national standard (GB/T 4789.2-1994). 25 g of sea cucumber samples were removed with sterile scissors and nippers, diluted in 225 ml physiological saline solution (PS, 0.87% NaCl, pH 7.0) and homogenized in a homogeniser (10 000 r.p.m.) for 1 min to obtain 10-fold dilute. 1 ml of the dilute was put into 9 ml PS to get 100-fold dilute, and then 1ml of that dilute was put into a sterile Petri dish. By the same method, 1 000-fold dilute was prepared and 1ml of it was put into a sterile Petri dish. All the dishes were injected with about 15 ml nutrient agar (46°C), then after solidification they were put into a cultivator, cultured for 48±2 h and counted. The real SPC should be the observed value multiplied by the dilution factor, and all the results were expressed in cfu g^{-1}. Detections were replicated three times for each sample.

Coliform bacteria is another indicator of food microbiological safety, and its detection method is described by the Chinese National Standard (Microbiological examination of food hygiene detection of *Coliform bacteria* (GB/T4789.3-2003)).

Smashing samples were weighed with accuracy of up to 0.2 g and detected with the graphite furnace atomic absorption after microwave digestion.

RESULTS AND DISCUSSION

The granularity distribution of the prepared nano-silver starch antibacterial film solution is shown in Fig. 1. The highest absorption peak was 60.81 nm, and the average diameter of the particles was 71.30 nm. The token result showed that the silver particles in nano-silver starch antibacterial film solution reached the nano-scale level.

It is no clear distinction of drying curve of the sea cucumbers with various coating (Fig. 2). This showed that the coating does not affect the drying rate. It had the same speed drying with dealing with different coating. It is no clear distinction of rehydration ratio curve of the sea cucumbers with various coating (Fig. 3). This showed that the coating does not affect the rehydration rate. Anyway, it have no effect on rates of drying and rehabilitation with nano-silver coating, whether coated with nano-silver solution, or coated with nano-silver antibacterial film starch solution.

The changes of the total number of bacteria in sea cucumber microwave-freeze dried for 9 h are shown in Fig. 4. The number of microorganisms decreased in the various pretreatments of sea cucumbers. The largest decline in the number of bacteria was noted for sea cucumber coated with nano-silver starch film (two orders of magnitude of microbial reduction and sterilization reached 99.1%, correspondingly, 95.3% for those coated with nano-silver solution, and 90.3% for the control group). Analysis of the effect of

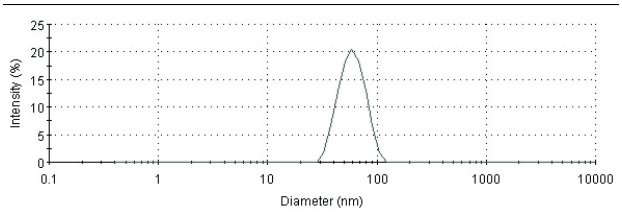

Fig. 1. Granularity distribution of nano-silver in starch soil.

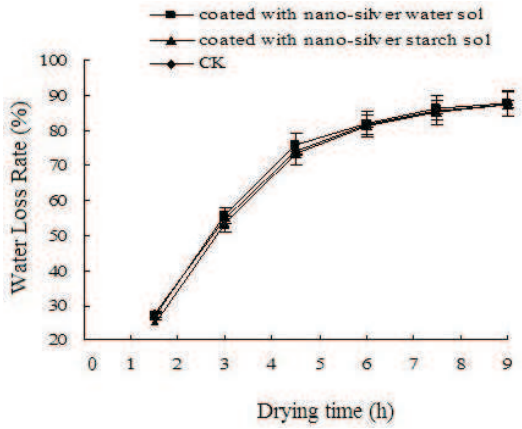

Fig. 2. Effect of coating on water loss (%) during drying.

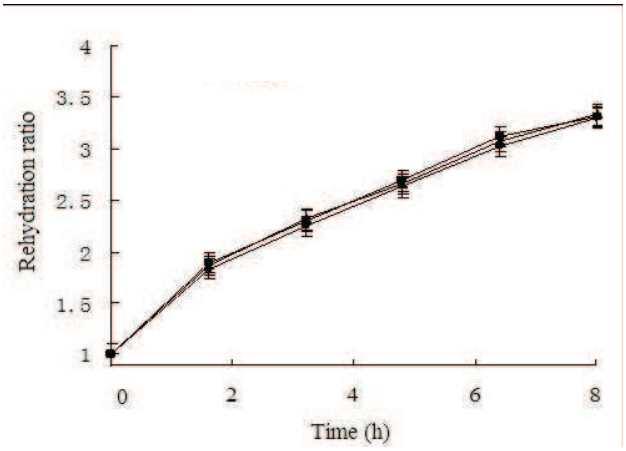

Fig. 3. Effect of coating on rehydration ratio during drying. Explanations as in Fig. 2.

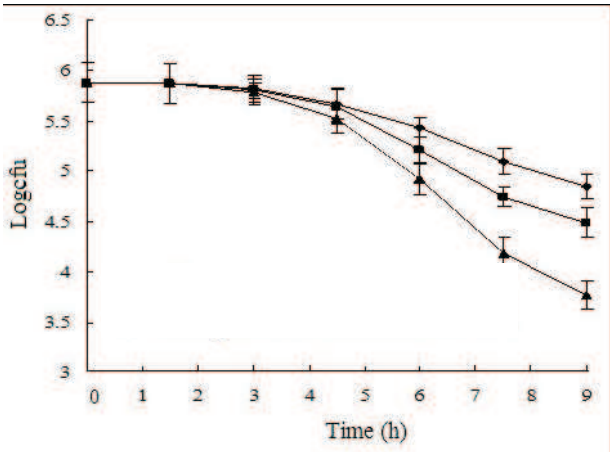

Fig. 4. Effect of nano-silver coating on aerobic bacterial count of sea cucumber during drying. Explanations as in Fig. 2.

nano-silver coating on the numbers of *Coliform bacteria* on sea cucumber showed that there were changes of the total number of *Coliform bacteria* in the sea cucumber with final water content below 3% after 9 h microwave-freeze drying. It can control *Coliform bacteria* within the scope of protective coating with nano-silver starch membrane solution (<30), compared with 50 for samples coated with nano-silver water solution and 96 for the control. The bactericidal effect of nano-silver starch film solution was significantly better than that of the nano-silver solution. The reasons are as follows. First of all, because the starch solution viscosity is greater than that of the water solution, even if sea cucumbers are coated with the same concentration of nano-silver, there are certain differences of the nano-silver content on sea cucumbers surface. Secondly, coating with the nano-silver starch solution forms a transparent film on the surface of sea cucumber, and this film layer can change the micro-environment of microbial life on the surface the sea cucumber which limits the growth of microorganisms. Thirdly, the layer of film on sea cucumbers surface can prevent the outside microbial life from direct contact with sea cucumbers surface.

The relationship between the bactericidal effect and the concentration of the nano-silver coating is shown in Fig. 5. With increased concentration, the number of surviving bacteria decreases gradually, the bactericidal effect of nano-silver is gradually enhanced, while the enhancing rate of sterilization with nano-silver starch antibacterial membrane is greater than that of the nano-silver solution.

The sterilization effects of nano-silver starch antibacterial film are stronger than those of the nano-silver coating solution in various concentrations. When coating with the nano-silver solution was applied, the bactericidal effect was not obvious when the concentration was in the range of 0.05~0.2 mg l^{-1}. There was little variation of the sterilization effect when different concentrations were used in the process of drying. No colony appeared in the microbial testing after the sea cucumbers were sterilized at 115°C during 15 min. There was no survival of bacteria in sea cucumber, basically.

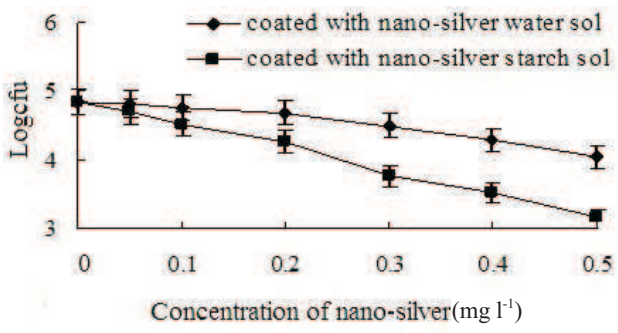

Fig. 5. Relation between concentrations of nano-silver and aerobic bacterial count (cfu).

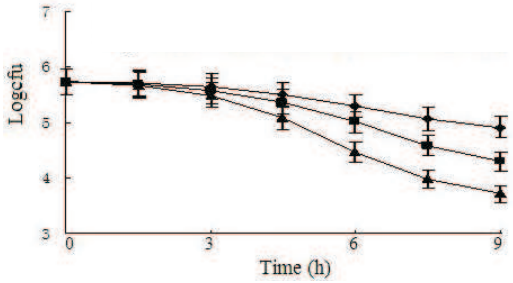

Fig. 6. Effect of nano-silver coating on *Bacillus subtilis* during drying (cfu). Explanations as in Fig. 2.

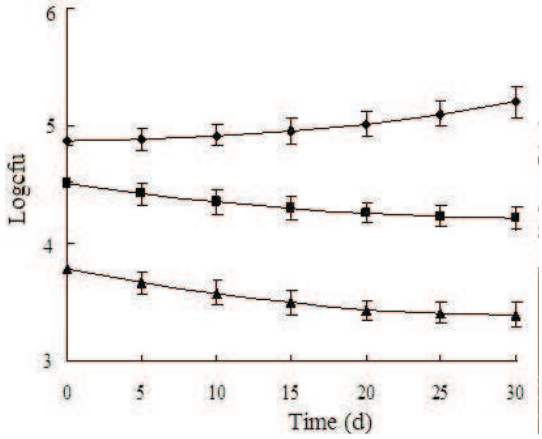

Fig. 7. Effect of microwave power of various stages during MFD process on sterilization. Explanations as in Fig. 2.

When the inoculation with *Bacillus subtilis* was applied, the sea cucumber contained *Bacillus subtilis* at a level of more than 10^5 cfu after training. Figure 6 shows the survival rate of *Bacillus subtilis* after the coating and microwave-freeze drying. Microwave has many aspects for sterilization; one important aspect is the microwave thermal effect. Because *Bacillus subtilis* can form spores, it is highly heat resistant. The sterilized sea cucumbers were inoculated with *Bacillus subtilis*, which permitted the study of nano-silver sterilization alone, excluding the thermal effect of the microwaves. As shown in Fig. 6, the mortality rate *Bacillus subtilis* in the control group was 85%, while rates of 94.3 and 99%, respectively, were obtained for sea cucumber coated with the nano-silver solution and the nano-silver starch solution in the process of microwave-freeze drying. Compared with the total number of bacteria, *Bacillus subtilis* has lower mortality in the process of microwave-freeze drying in the control group.

The balanced moisture content of sea cucumber was 51% at 30°C, and relative humidity was 92.31%, regulated by the use of KNO_3 saturated solution. At the same time, the water activity of sea cucumber was 0.92. Changes of the total number of bacteria in sea cucumber stored for one month at 30°C and relative humidity 92.31% are presented in Fig. 7. The total numbers of bacteria increased on the sea cucumber of the control group, and were reduced on the sea cucumber coated with the nano-silver solution and the nano-silver starch solution. The rate of decline in the total number of bacteria was faster in the early stages of storage, and basically stabilized later on in the storage period. The rate of decline and the range of the total numbers of bacteria were greater for the group of samples coated with the nano-silver starch film than for the group in which the nano-silver water solution was applied on sea cucumbers.

As far as the moisture absorption properties of MFD sea cucumber and the stability of the antibacterial properties of nano-silver are concerned, the number of microbes continued to increase in the control group a month later, but there was a plateau period before a slow increase in the microbial count in the case of sea cucumber coated with nano-silver.

The results of atomic absorption test showed the content of silver on MFD sea cucumber. The content of silver in MFD sea cucumber was very few, only 0.165±0.008 mg kg^{-1} for samples coated with the nano-silver water solution and 0.177±0.009 mg kg^{-1} for those coated with the nano-silver starch solution, so coating with nano-silver is a safe sterilization technology. The content of silver would be much less still after the rehydration and cleaning of sea cucumbers before cooking.

CONCLUSIONS

1. The particle size of nano-silver which was prepared by the liquid phase chemical deoxidation method was small enough and stable in the starch solution.

2. There was no effect of nano-silver coating on the rates of drying and rehydration, whether coating with the nano-silver solution or with the nano-silver antibacterial film starch solution was applied.

3. The reduction of the number of microorganisms on sea cucumber reached 99.1% using coating with nano-silver starch film and 95.3% using coating with nano-silver solution, compared with 90.3% for the control group. Thus, coating with nano-silver starch membrane solution can provide control of *Coliform bacteria* at the required level of food safety.

4. The mortality rates of *Bacillus subtilis* reached 94.3% and 99% using coating with nano-silver solution and nano-silver starch solution in the process of microwave-freeze drying of sea cucumbers, compared with 85% in the control group.

5. The total number of bacteria increased in the control group of sea cucumbers and decreased on sea cucumbers coated with nano-silver solution and nano-silver starch solution during storage for one month at 30°C and 92.31% relative humidity.

6. The silver content of on MFD sea cucumber was very low at only 0.165 mg kg^{-1} in the case of coating with nano-silver solution, and 0.177 mg kg^{-1} when coating with nano-silver antibacterial film starch solution was applied, so coating with nano-silver is a safe microbial control technology.

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