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Studies on the Development of a Microbial Cryoprotectant Formulation Using a W/O/W Multiple Emulsion System

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Abstract A microbial cryoprotectant formulation using a W/O/W multiple emulsion system was developed. The psychrotolerant microorganism, B4, isolated from soil in South Korea, was observed by the drop freezing method, in which the microorganism sample inhibited ice nucleation activity. The antifreeze activity was eliminated when the microorganism sample was treated with protease, indicating that the antifreeze activity was due to the presence of antifreeze protein. The result of the 16S rDNA sequencing indicated the B4 strain was most closely related to a species of the genus *Bacillus*. Culture broth of B4 strain (*Bacillus* sp.) and rapeseed oil containing 1% polyglycerine polyricinolate (PGPR) were used as core and wall material, respectively. The most stable W/O emulsion was prepared at a core/oil ratio of 1:2. The highest W/O/W emulsion stability was achieved when the primary emulsion to external aqueous phase containing 0.5% caster oil polyoxyethylene ether (COG25™) ratio was 1:1. Microcrystalline cellulose showed better W/O/W emulsion stability than other polymer types. The viability of cells in a W/O/W emulsion was higher than free cells during storage at 37°C. An acidic pH and UV exposure decreased the viability of free cells, but cells in W/O/W emulsion were more stable under these conditions.

Key words: Antifreeze protein, microbial cryoprotectant, W/O/W emulsion

Several microorganisms produce an ice nucleation protein that is apparently located on the surface of the bacterial cell. This protein causes formation of ice at temperatures only slightly below 0°C and subsequently inflicts significant frost damage on important plants. Plant productivity can be affected by bacteria that incite frost injury [18, 28].

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The inhibitory effect of an antifreeze protein on bacterial ice nucleators is known [23]. In nature, many organisms are exposed to freezing temperature. An interesting adaptation to life at subzero temperatures is the production of proteins that modify ice crystal growth. Antifreeze proteins have the ability to inhibit the growth of ice crystals and ice nucleation protein activity [9, 12, 22]. These proteins have been isolated in a wide range of organisms including microorganisms [8, 27, 30], plants [2, 8, 13, 14, 18, 29], invertebrates [11], and fish [5, 6, 22].

Nowadays, many have been made for biological control using microorganisms as an alternative tool for chemical control [15, 16, 19, 31]. In addition, many recent studies focused on a biological control mechanism to decrease plant frost damage using microorganisms as a tool [17, 20]. However, a little research on the antifreeze proteins produced by microorganisms has been reported. Multiple forms of antifreeze proteins may be synthesized by different species of microorganisms. Therefore, it is possible to select an antifreeze protein with appropriate characteristics and a suitable level of activity for a particular biological control application to reduce frost damage.

In this study, the main concern was to use the microorganism that is sprayed on plants and which might prevent frost damage by inhibiting and displacing the common, ice-promoting kind. However, to control frost damage, the use of a microorganism having antifreeze protein activity as cryoprotectant is accompanied by problems including microbial degradation and a decrease in the residual activity of the protein after application in the field due to environment conditions, including sun light, high temperature, leaf surface exudates, and rain. Formulations aid organism preservation and helps to deliver the organism to the target, thus improving organism activities. Formulations of organisms can be broadly divided into solid and liquid groups [3]. Dry formulations have limitations. Preparation of dry formulations involves complex technology, is time

consuming, and less cost effective than liquid formulations. Of liquid formulations, a suspension of microbial agent in water would be the simplest spray formulation. However, this formulation would seldom be effective under practical conditions because of factors such as leaf waxes that resist wetting and even distribution of the agent. Many studies have shown that a formulation in oil enhances the activity of the agent compared with a conventional water-based formulation [1, 3, 17]. Therefore, a microbial cryoprotectant formulation using a W/O/W (water-in-oil-in-water) multiple emulsion system was developed in an effort to overcome these limitations. A W/O/W multiple emulsion is a system capable of entrapping beneficial substances in an internal aqueous compartment. The main application of this technology is to protect entrapped substances and control their release into the external aqueous phase [10]. Emulsions were used as carriers of active ingredients in agriculture applications to formulate active substances for treatment of plants and soil [4].

The objective of this investigation was to explore a new microbial cryoprotectant formulation using a W/O/W multiple emulsion system to provide effective frost damage prevention.

Materials and Methods

Materials

Rapeseed (CJ Co., Seoul, South Korea) oil was used as wall material. The emulsifiers used for stabilizing the emulsion were the hydrophobic emulsifier polyglycerin polyriconolate (PGPR, Ilshin Emulsifier Co., Seoul, South Korea), and the hydrophilic emulsifier castor oil polyoxyethylene ether (COG 25, Coseal Co., Kunsan, South Korea). The hydrophilic polymers used for stabilizing the W/O/W multiple emulsion system were alginate (Showa Chem. Inc., Osaka, Japan), carrageenan (Viscarin®SD 389, FMC Co., Cebu, The Philippines), microcrystalline cellulose (Avicel-plus™CG 200, FMC Co., Cebu, The Philippines), gelatin (Sammi Industrial Co., Ansan, South Korea), and pectin (PECTIN AMID AF 020-A, Herbstreith&Fox, Neuenbürg, Germany).

Microorganism and Culture Conditions

The B4 strain was isolated from a soil sample collected near Kanghwa, South Korea in early March. The organism was cultured on nutrient agar plate for 72 h at 4°C. For long-term maintenance, a cell suspension was stored at -40°C in broth cultures supplemented with 20% (v/v) glycerol.

Antifreeze Activity Assay

Samples were prepared for screening microorganisms with antifreeze activity. Strains isolated from soil were inoculated into a nutrient broth. After incubation at 4°C for 3-4 days, the culture broth was centrifuged at 6,000 rpm for 15 min at 4°C. The supernatant was concentrated by ultrafiltration using an Amicon YM3 membrane with a nominal molecular weight cutoff of 3,000 Da (Millipore Co., U.S.A.). The antifreeze activity inhibiting bacterial ice nucleator was examined using the drop freezing method [21]. An active sample was treated with a nonspecific bacterial protease from *Streptomyces griseus* (Sigma Chemical Co., Type VI bacterial protease) and checked for loss of antifreeze protein activity to determine whether the antifreeze activity resulted from the presence of a protein [8].

Identification of Microorganism

Cell morphology was examined by light microscopy. For 16S rDNA sequence analysis, amplification of 16S rDNA by PCR was performed using the primers 9F 5' GAG TTT GAT CCT GGC TCA G 3' and 1542 R 5' AGA AAG GAG GTG ATC CAG CC 3' corresponding to positions 9-27 and 1542-1523, respectively, in the 16S rRNA sequence of *Escherichia coli*. The amplified PCR product was directly ligated into the pGEM-T Easy vector system II (Promega®, U.S.A.). Clones were screened and plasmid DNA was purified by the Minipreps DNA purification system (Promega®, U.S.A.). Sequencing was performed using the T7 primer 5'-TAATACGACTCACTATAGGG-3' and the M13 reverse primer 5'-GGATAACAATTTCAC-ACAGG-3'.

Preparation of Emulsion

Culture broth of the B4 strain and rapeseed oil containing 1% polyglycerin polyriconolate were used as core and wall materials, respectively. A primary W/O emulsion was prepared by adding the culture broth to the oil phase. This mixture was homogenized for 10 min at 3,500 rpm using a homomixer (Polytron PT-3000, Kinematica, Japan) to form a W/O emulsion. The stability of the W/O emulsion was tested by changing the ratio of internal aqueous to the oily phase from 1:1, to 1:2, and finally to 2:1. A W/O/W multiple emulsion was prepared using a homomixer for 15 min at 2,500 rpm. The primary emulsion was dispersed into the aqueous phase containing the hydrophilic emulsifier, 0.5% castor oil polyoxyethylene ether, and a stabilizer. The effects of changing the ratio of the primary emulsion to the external aqueous phase, the stabilizer type, and the concentration of stabilizer on emulsion stability were investigated. All experiments were carried out in triplicate and all data represent the mean value of three replicates.

Measurement of Emulsion Stability

A sample of the liquid emulsion was placed in a 10 ml mass cylinder that was capped and subjected to accelerated aging in a 55°C oven. After phase separation, the volume of the translucent aqueous phase was read. Results are presented as an emulsion stability index (ESI) ranging

from 0 to 1 with a value of 0 representing poor emulsion stability.

$$ESI = 1 - \frac{\text{Total volume of separated water}}{\text{Total volume of water in emulsion}}$$

Morphology Analysis of Droplets in the Emulsion

The morphology of droplets in the W/O primary emulsion and the W/O/W multiple emulsion was observed under a light microscope (BHT 312, Olympus Optical Co., Tokyo, Japan).

Storage Stability of W/O/W Emulsion

Free cells and cells in the W/O/W multiple emulsion system were stored at 37°C. Cell viabilities for both were monitored over 10 weeks at intervals of 1 week by counting the number of colonies.

Stability Against pH Treatments and UV Radiation

The tolerance of cells under various pH conditions and UV radiation was determined [7]. pH treatments were performed by mixing samples of free cells and W/O/W emulsion with buffers of pH values between 3 and 10. The number of colonies was counted after 1 h at 25°C. Stability against UV radiation was assayed by exposing samples to a UV lamp and counting colonies at 24-h intervals for 72 h.

RESULTS AND DISCUSSION

Isolation of Microorganism

Screening from soil produced 12 isolates that were capable of growth at close to 0°C. These isolates were tested for an ability to grow at a lower temperature. Strain B4, which grew at the lowest temperature, was selected for further study. According to cumulative ice nucleation activity spectra observed by the drop freezing method, a sample of B4 showed an inhibitory effect on ice nucleation activity (Fig. 1). The antifreeze activity was eliminated when the B4 sample was treated with protease, indicating that the antifreeze activity was due to the presence of an antifreeze protein (Fig. 2).

Identification of Microorganism

Strain B4 was Gram-positive, rod shaped, and formed circular, mucous, and white colonies. An almost complete 16S rDNA sequence (>1,500 nt) was determined. BLAST searches of the 16S rDNA sequence indicated the strain was most closely related to species of the genus *Bacillus*. The 16S rDNA of B4 showed sequence similarities of 99% to *Bacillus simplex* and 97% to *Bacillus psychrosaccarolyticus* (Table 1). Although strain B4 showed high sequence similarity to *Bacillus simplex* and

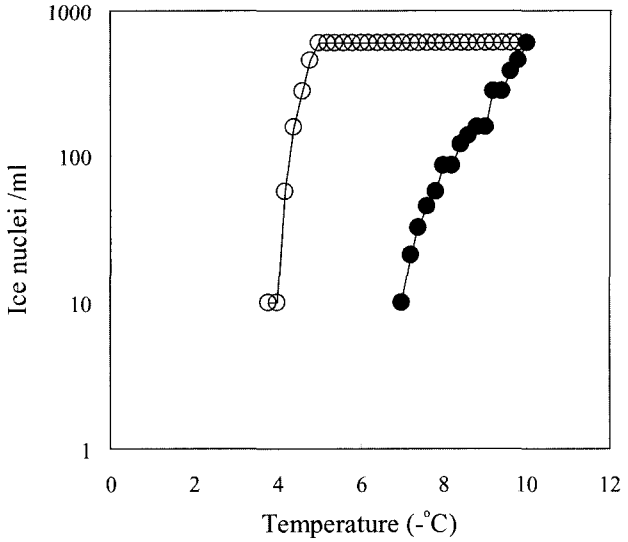

Fig. 1. Comparison of a cumulative ice nucleation activity spectrum of an antifreeze-active strain.
-○-: control (bacterial ice nucleator); -●-: antifreeze active strain (bacterial ice nucleator+B4).

Bacillus psychrosaccarolyticus, further taxonomic study of B4 is needed, because the strain exhibited physiological properties that were different from *Bacillus simplex* and *Bacillus psychrosaccarolyticus* [24, 26].

Stability of the Emulsion

Stability of the primary emulsion is necessary for successful stabilization of a multiple emulsion [25]. The effect of

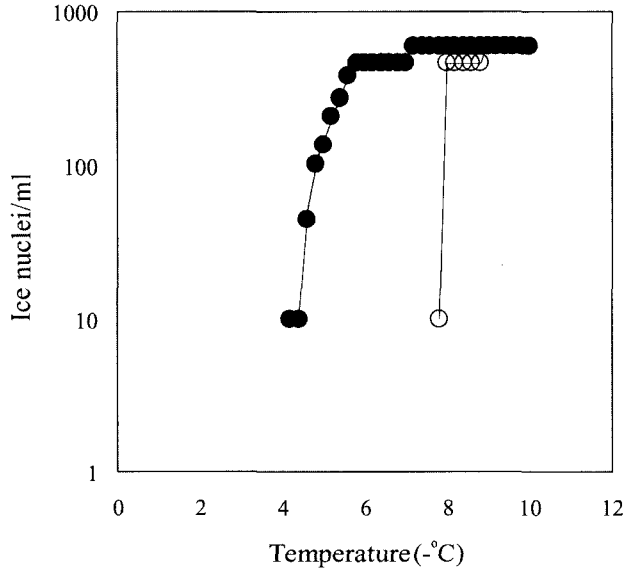

Fig. 2. Comparison of an antifreeze protein activity assay after protease treatment.
-○-: protease nontreatment; -●-: protease treatment.

Table 1. Similarities of the B4 strain to known taxa based on the result of 16s rDNA sequencing.

Strain	Identities (%)
Bacillus simplex	1515/1523 (99%)
Bacillus psychrosaccharolyticus	1418/1450 (97%)
Bacillus niacini	1468/1528 (96%)
Bacillus flexus	1462/1529 (95%)
Bacillus benzoevorans	1455/1526 (95%)
Bacillus fumarioli	1437/1505 (95%)
Bacillus megaterium	1426/1498 (95%)

the dispersed phase (core) to continuous phase (oil) ratio on the stability of the W/O primary emulsion was determined. The most stable ratio was 1:2 (Fig. 3) and the highest W/O/W emulsion stability was achieved when the primary emulsion to the external aqueous phase ratio was 1:1 (Fig. 4). W/O/W multiple emulsions are thermodynamically unstable. Therefore, maintaining emulsion stability requires an emulsifying agent. Wash-off is one of the factors that affects the field persistence and activity of the biocontrol agents. Stabilizer improves adherence of organisms to foliage and persistence during rain. Polymers have been used to stabilize W/O/W multiple emulsions [10] and enhance adherence of the multiple emulsion [3, 10], so various hydrophilic polymers were tested. MCC resulted in better emulsion stability than other polymer types (Fig. 5). The stability of the emulsion increased with an increasing MCC concentration (Fig. 6). The composition that was selected for preparation of the W/O/W multiple emulsion formulation is shown in Table 2.

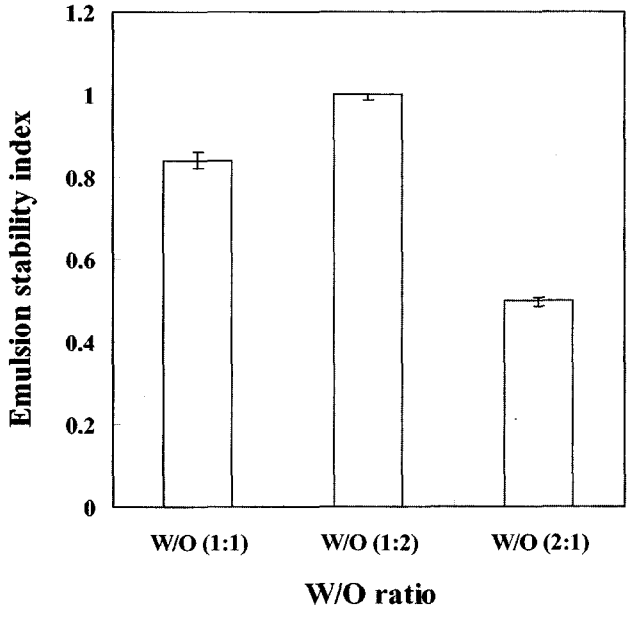

Fig. 3. Effect of various W/O ratios on the emulsion stability.

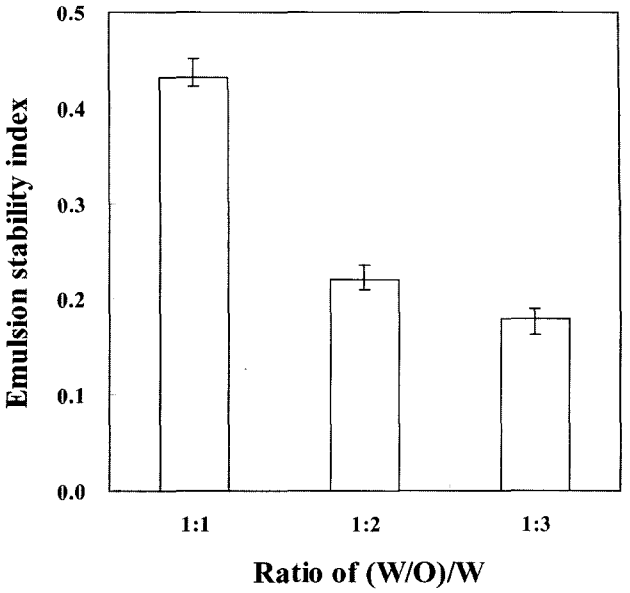

Fig. 4. Effect of various core/oil ratios on the stability of the W/O/W multiple emulsion.

Morphology Analysis of Droplets in the Emulsion

W/O emulsion droplets were observed under a light microscope (Fig. 7A). Droplets of culture broth containing B4 cells were entrapped by the oil layer with a diameter of approximately 2-8 μm. Droplets separated from the aqueous dispersion phase by the oil phase were observed in the W/O/W multiple emulsion with a diameter of approximately 10-100 μm.

Storage Stability of Cells in the W/O/W Emulsion

The cell viability is important because biocontrol agents are required to maintain the viability of cells during the

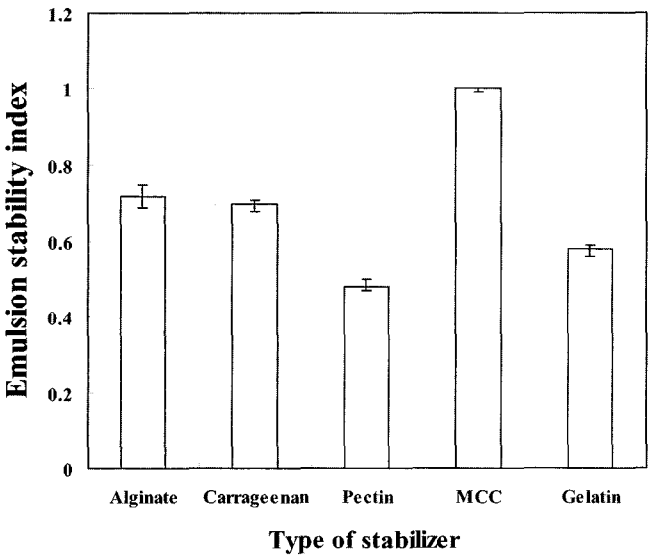

Fig. 5. Stability of W/O/W multiple emulsion with various stabilizers.

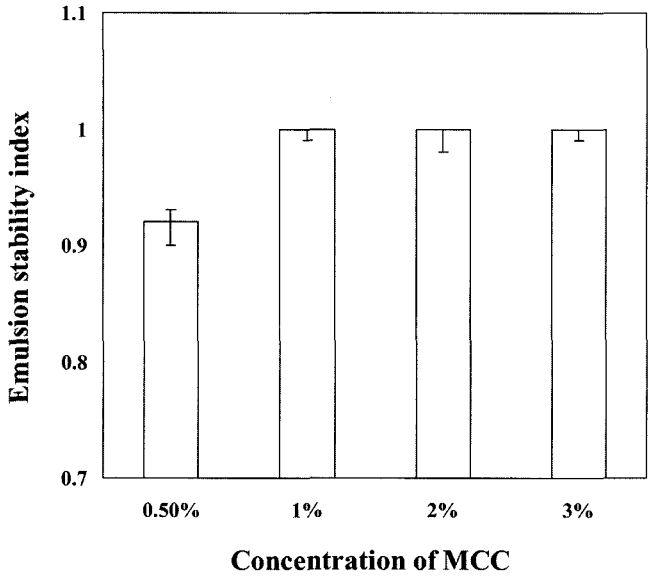

Fig. 6. Effect of the microcrystalline cellulose concentration on the stability of the W/O/W multiple emulsion.

storage period, and the time between manufacture and eventual use in the field [3]. Also, there is a direct relation between protein production and cell viability, so production of antifreeze protein increases with an increasing cell viability. Therefore, the storage stability of cells in the W/O/W multiple emulsion was tested. The viability of free cells during storage at 37°C was reduced by 4 log cycles (10^4) while the viability of cells in the W/O/W multiple emulsion decreased by 1 log cycle (10^1) by the end of storage (Fig. 8). In this study, the viability of cells in the W/O/W multiple emulsion was higher than for free cells during storage, probably because cells in the W/O/W multiple emulsion had a slower metabolic rate than free cells. Limited aeration of cells in the W/O/W multiple emulsion caused inhibited nutrient depletion and reduced accumulation of toxic metabolites.

Stability Against pH Treatments and UV Radiation

Acidic pH values and UV exposure decreased the viability of free cells, while cells in the W/O/W multiple emulsion were more stable under these conditions. Cells in the W/O/W multiple emulsion were more stable under acidic conditions (Fig. 9), probably due to lack of direct contact of the microorganism with the acidic environment. Solar inactivation

Table 2. Composition of the W/O/W multiple emulsion.

	Component	Weight % in W/O/W emulsion
W/O primary emulsion	Culture broth	16.6
	Rapeseed oil	33.1
	PGPR	0.3
W/O/W multiple emulsion	COG25	2.2
	MCC	4.3
	Distilled water	43.5

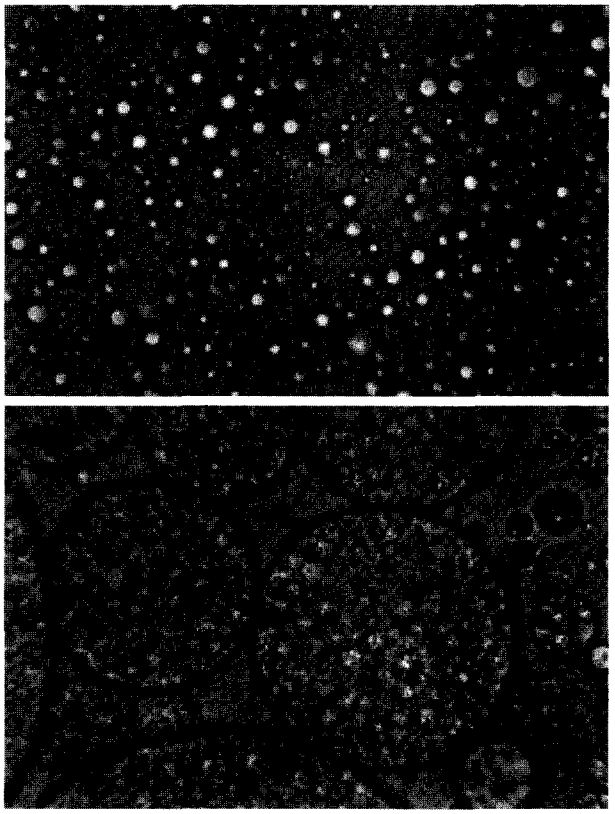

Fig. 7. Photomicrographs of a W/O primary emulsion and a W/O/W multiple emulsion (magnification ×1,000). Bar, 15 μm.

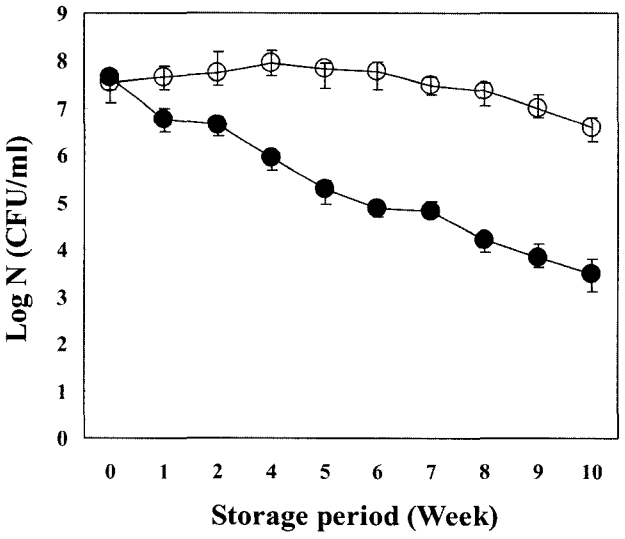

Fig. 8. The viability of cells in the W/O/W multiple emulsion during storage at 37°C.
-○-: 37°C control; -●-: 37°C W/O/W multiple emulsion.

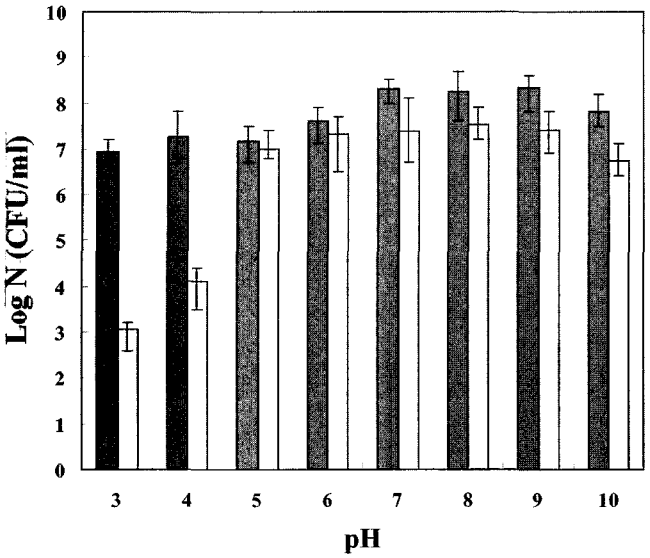

Fig. 9. Effect of different pH values on the viabilities of free cells and cells in the W/O/W multiple emulsion.
□: free cell; ■: W/O/W multiple emulsion.

of biological control agents is a widely recognized phenomenon. Exposure to light with a wavelength below 500 nm for short period inactivates microorganisms and degrades proteins produced by microorganisms [3]. Current observations showed that the viability of cells in the W/O/W multiple emulsion was higher after UV irradiation than that of free cells. The viability of free cells was reduced by 1.5 log cycles while that of cells in the W/O/W multiple emulsion decreased by only 0.8 of a log cycle (Fig. 10). This result confirms that the vegetable oil is able to protect against the injurious effect of sunlight as shown in other studies [1, 18].

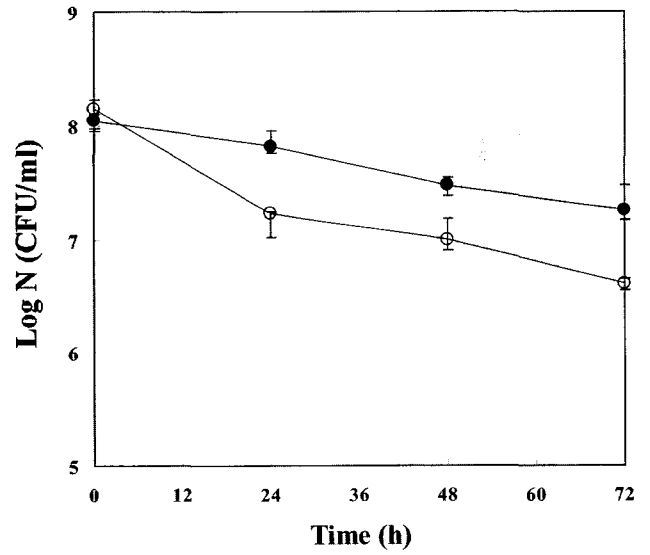

Fig. 10. Effect of UV radiation on the stability of free cells and cells in the W/O/W multiple emulsion.
-○-: free cell; -●-: W/O/W multiple emulsion.

Conclusion

Formulation of a W/O/W multiple emulsion is attractive because it is simple and the ingredients are readily available. The process can be adopted for other types of biocontrol agents without major modifications. The high viability of microorganism having antifreeze protein activity in the W/O/W multiple emulsion indicates that the formulation of W/O/W multiple emulsion can be used effectively in the field as a microbial cryoprotectant. Further studies are underway regarding the field applicability of this W/O/W multiple emulsion system.

REFERENCES

1. Alves, R. T., R. P. Bateman, C. Prior, and S. R. Leather. 1998. Effect of simulated solar radiation of conidial germination of *Metarhizium anisopliae* in different formulations. *Crop Protect.* **17:** 675-679.
2. Boothe, J. G., F. D. Sonnichsen, M. D. Beus, and A. M. Johnson-Flanagan. 1997. Purification, characterization, and structural analysis of a plant low-temperature-induced protein. *Plant Physiol.* **113:** 367-376.
3. Burges, H. D. 1998. *Formulation of Microbial Biopesticides*, pp. 2-27. Kluwer Academic Publishers. Dordrecht. The Nethelands.
4. Chappat, M. 1994. Some application of emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **91:** 57-77.
5. Cheng, A. and K. M. Merz. 1997. Ice-binding mechanism of winter flounder antifreeze proteins. *Biophys. J.* **73:** 2851-2873.
6. Cheng, C. C. and A. L. DeVries. 1989. Structures of antifreeze peptides from the antarctic eel pout, *Austrolycicthys brachycephalus*. *Biochim. Biophys. Acta* **997:** 55-64.
7. Cokmus, C. and Y. M. Elcin. 1995. Stability and controlled release properties of carboxymethylcellulose-encapsulated *Bacillus thuringiensis* var. *israelensis*. *Pestic. Sci.* **45:** 351-355.
8. Duman, J. G. and T. M. Olsen. 1993. Thermal hysteresis activity in bacteria, fungi, and phylogenetically diverse plants. *Cryobiology* **30:** 322-328.
9. Feeney, R. E. and Y. Yeh. 1993. Antifreeze proteins: Properties, mechanism of action, and possible application. *Food Technol.* 83-88.
10. Garti, N. 1997. Progress in stabilization and transport phenomena of double emulsions in food applications. *Lebensm.-Wiss. u-Technol.* **30:** 222-235.
11. Graham, L. A., Y. C. Lieu, V. K. Waker, and P. L. Davies. 1997. Hyperactive antifreeze protein from beetles. *Nature* **388(21):** 727-728.
12. Griffith, M. and K. V. Ewart. 1995. Antifreeze proteins and their potential use in frozen foods. *Biotech. Adv.* **13(3):** 375-402.

13. Griffith, M., M. Antikainen, W. C. Hon, P. M. Maunsbach, X. M. Yu, J. U. Chun, and D. S. C. Yang. 1997. Antifreeze proteins in winter rye. *Physiologia Plantarum* **100:** 327-332.
14. Griffith, M., P. A. Daniel, D. S. C. Yang, W. C. Hon, and B. A. Moffatt. 1992. Antifreeze protein produced endogenously in winter rye leaves. *Plant Physiol.* **100:** 593-596.
15. Kim, J. W., J. G. Kim, B. K. Park, O. H. Choi, C. S. Park, and I. G. Hwang. 2003. Identification of genes for biosynthesis of antibacterial compound from *Pseudomonas fluorescens* B16 and its activity against *Ralstonia solanacearum*. *J. Microbiol. Biotechnol.***13:** 292-300.
16. Kim, Y. S., H. S. Lim, and S. D. Kim. 1994. *Bacillus subtilis* YB-70 as a biocontrol agent of *Fusarium solani* causing plant root-rot. *J. Microbiol. Biotechnol.* **4:** 68-74.
17. Klein, T. A., B. A. Auld, and Fang Wang. 1995. Evaluation of oil suspension emulsions of *Colletotrichum orbiculare* as a mycoherbicide in field trials. *Crop Protect.* **14:** 193-197.
18. Lindow, S. E. 2002. Phyllosphere microbiology. *Curr. Opin. Biotech.* **13:** 238-243.
19. Lollford, P. J. and C. B. Holt. 1994. Antifreeze proteins. *J. Food Eng.* **22:** 475-482.
20. Ng, N. F. L. and C. L. Hew. 1992. Structure of an antifreeze polypeptide from the sea raven. *J. Biol. Chem.* **267(23):** 16069-16075.
21. Parody-Morreale, A., K. P. Murphy, E. D. Cera, R. Fall, A. L. DeVries, and S. J. Gill. 1988. Inhibition of bacterial ice nucleators by fish antifreeze glycoproteins. *Nature* **33(23):** 782-783.
22. Priest, F. G., M. Goodfellow, and C. Todd. 1988. A numerical classification of the genus *Bacillus*. *J. General Microbiol.* **134:** 1847-1882.
23. Schugens, Ch., N. Laruelle, N. Nihant, Ch. Grandfils, R. Jérome, and Ph. Teyssié. 1994. Effect of the emulsion stability on the morphology and porosity of semicrystalline poly L-lactide microparticles prepared by W/O/W double emulsion-evaporation. *J. Control Release* **32:** 161-176.
24. Sneath, P. H. A. 1957. *Bacillus*, pp. 1105-1139. *In* Claus, D. and Berkeley, R. C. W. (eds.), 7th Ed. *Bergey's Manual of Determinative Bacteriology.* Williams & Wilkins, Baltimore, U.S.A.
25. Sun, X., M. Griffith, J. J. Pasternak, and B. R. Glick. 1995. Low temperature growth, freezing survival, and production of antifreeze protein by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can. J. Microbiol.* **41:** 776-784.
26. Testolin, R. and G. Costa. 1995. Ice nucleation temperatures in kiwifruit. *Sci. Hortic.* **61:** 29-36.
27. Urrutia, M. E., J. G. Duman, and C. A. Knight. 1992. Plant thermal hysteresis proteins. *Biochim. Biophys. Acta* **1121:** 199-206.
28. Xu, H., M. Griffth, C. L. Patten, and B. R. Glick. 1998. Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium *Pseudomonas putida* GR 12-2. *Can. J. Microbiol.* **44:** 64-73.
29. Yoon, C. S., K. W. Yu, S. H. Bae, H. H. Song, H. S. Park, and C. Lee. 2003. Chemical properties and physiological activities of synnemata of *Beauveria bassiana*. *J. Microbiol. Biotechnol.* **13:** 125-133.