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Development of W/O/W Multiple Emulsion Formulation Containing *Burkholderia gladioli*

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Abstract W/O/W (water-in-oil-in-water) type multiple emulsion was applied to improve the storage stability of an antagonistic microorganism, *Burkholderia gladioli*. Encapsulation of microorganism into a W/O/W emulsion was conducted by using a two-step emulsification method. W/O/W emulsion was prepared by the incorporation of *B. gladioli* into rapeseed oil and the addition of polyglycerin polyriconolate (PGPR) and castor oil polyoxyethylene (COG 25) as the primary and secondary emulsifier, respectively. Microcrystalline cellulose was used as an emulsion stabilizer. To evaluate the usefulness of W/O/W emulsion formulation as a microbial pesticide for controlling the bacterial wilt pathogen (*Ralstonia solanacearum*), the storage stability and antagonistic activity of emulsion formulation were tested *in vitro*. The storage stability test revealed that the viability of formulated cells in emulsion was higher than that of unformulated cells in culture broth. At 4°C, the viabilities of formulated cells and unformulated cells at the end of 20 weeks decreased to about 2 and 5 log cycles, respectively. At 37°C, the viability of formulated cells decreased to only 2 log cycles at the end of storage. On the other hand, the viable cells in culture broth were not detected after 13 weeks. In activity test, formulated cells in emulsion were more effective in inhibiting the growth of pathogen than unformulated cells in culture broth. Unformulated cells completely lost their antagonistic activity during storage under similar conditions. The W/O/W multiple emulsion formulation was shown to be useful as the novel liquid formulation for biological control.

Key words: W/O/W multiple emulsion formulation, antagonistic microorganism, *Burkholderia gladioli*, storage stability, antagonistic activity

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Biological control agents to plant diseases are currently being examined as alternatives to chemical control, because of their perceived high level of safety and minimal environmental impacts [3, 6, 9, 10, 12, 13, 14, 27]. However, the shelf and field lives of most microorganisms used as biological control agents (BCAs) are generally short compared with chemical agents. For this reason, many BCAs have been formulated into various form of liquids, solids, and powders [19], which contribute to higher viability and stability in storage, optimum application to the target, and easy handling of the product by users. Formulation of BCAs for commercial use involves the mixing of BCA inoculums with carrier substances. Most of the recently patented and published researches on the formulation of BCAs, including fungi, bacteria, and nematodes, deal with methods for preparing solid (dry) formulations such as powder, granule, and pellet [2, 17, 19, 20, 21, 24, 25, 26]. It has been that the shelf life of dry formulations of BCAs can be increased by adding alternative bulking agents or nutrient adjuvants.

There are a wide variety of formulation types, both liquid and solid [5]. The main types currently used for organisms have been classified by Rhodes into dry products (dusts, granules, and briquettes) and suspensions (oil- or water-based and emulsions). In practice, dry formulations are generally preferred over liquid formulations, because they provide extended shelf life and ease in transportation and storage. Furthermore, most granular or powder formulation can be made into water-based suspension as required for drench, spray, or root-dip applications [19].

However, these dry formulations have some limitations. Preparation of dry formulations involves complex technology, thus, is time consuming and less cost effective than that of liquid formulations in terms of manufacturing process.

One of the serious problems associated with dehydration of granules containing living organism, using large-scale equipment such as fluidized bed dryer, is that granules tend to dry out quickly, which is harmful to their survival since moisture is necessary to maintain the vitality of organisms for extended storage periods. In addition, dried granules are very difficult to dissolve in water; only partial dissolution is achieved. It appears that this product is limited to direct soil application and cannot be used in spray application, due to submerging of granules in water. As described above, the conventional methods for formulating microbial biopesticides could not technically and economically provide more feasible pesticides. In this study, we attempted to develop a novel liquid formulation, W/O/W (water-in-oil-in-water) multiple emulsion, as an alternative method to overcome the above problems of solid formulation. W/O/W multiple emulsion is a system able to entrap beneficial substances in the internal aqueous compartment [22]. Its main application is to protect the entrapped substances and control their release into the external aqueous phase. This system is comprised of three phases; an internal aqueous phase containing beneficial substances, an oil phase surrounding it, and an external aqueous phase around the oil phase. Thus, this complex system has shown promise in many technologies, particularly in pharmaceutics [8, 16, 23], and also in nonpharmaceutical areas for slow and controlled release of materials such as fertilizers and pesticides for agricultural uses, as well as for cosmetic industries and food application [7].

An antagonistic bacterium, *Burkholderia gladioli* (*Pseudomonas gladioli*), isolated from soil in Korea, was used as a BCA to formulate into W/O/W emulsion. The strain showed strong inhibitory effects on growth of *Ralstonia solanacearum* (*Pseudomonas solanacearum*), a wilt-inducing pathogen of plants, including tomato, potato, eggplant, and sweet pepper [15]. However, possibilities for commercialization are limited, because it is difficult to control the quality of the final product. W/O/W emulsion was applied to improve the storage stability of *B. gladioli*. The objectives of this research were to formulate antagonistic bacterium, *B. gladioli*, using stable W/O/W emulsion and to examine the storage stability and the antagonistic activity of cells in the formulation during storage under laboratory conditions.

MATERIALS AND METHODS

Materials

The emulsifiers used as the primary and secondary emulsifier were polyglycerin polyriconolate (PGPR, Ilshin Emulsifier Co., Seoul, South Korea) and castor oil polyoxyethylene ether (COG 25, Coseal Co., Kunsan, South Korea). The rapeseed oil was purchased from CJ Co. (Seoul, South Korea). The rapeseed oil was selected for this study, because it showed higher oxidation stability than other vegetable oils. Five hydrophilic polymer materials used for stabilizing multiple emulsions were alginate (Showa Chem. Inc., Osaka, Japan), carrageenan (Viscarin®SD 389, FMC Co., Cebu, Philippines), microcrystalline cellulose (Avicel-plus™CG 200, FMC Co., Cebu, Philippines), gelatin (Sammi Industrial Co., Ansan, South Korea), and pectin (PECTIN AMID AF 020-A, Herbstreith & Fox, Neuenbürg, Germany).

Microorganism and Culture Conditions

Burkholderia gladioli strain was provided by Green Biotech Co. Ltd (Paju, South Korea). To obtain culture broth containing fresh cells for formulation, *B. gladioli* was grown on liquid medium (20 g peptone, 10 ml glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 1 l distilled water, pH 7.0) for 24 h in a shaking incubator (200 rpm) at 30°C. Culture broth obtained was stored at 4°C and used directly as an internal aqueous phase (core material) of W/O/W emulsion.

Preparation of Emulsion Formulation

The composition selected for preparation of W/O/W emulsion formulation in this study is shown in Table 1. W/O/W emulsion was prepared by a two-step emulsification procedure at room temperature (25±2°C), using a homomixer (Polytron PT-3000, Kinematica, Japan). Before the manufacturing of emulsion, all glassware and solutions were sterilized at 121°C for 15 min to prevent unwanted contamination. Culture broth containing viable cells of 5×10^9 colony-forming units (CFU)/ml was used as the internal aqueous phase (core material) of W/O/W emulsion. The rapeseed oil containing 1% hydrophobic emulsifier (PGPR) was used as the oil phase. In the first step, W/O primary emulsion was formed by adding the culture broth into oil phase at 1:2 ratio (by weight). The mixture was homogenized for 10 min at 3,500 rpm, using

Table 1. Composition of the W/O/W multiple emulsion.

Step	Constituents		Concentration wt%
W/O emulsion	W1 phase (Internal aqueous phase)	Culture broth	16.67
	Oil phase	Rapeseed oil	33.00
		PGPR	0.33
W/O/W multiple emulsion	W2 phase (External aqueous phase)	Distilled water	48.75
		COG 25	0.50
		MCC	0.75

a homomixer. Prior to the second step of emulsification, the external aqueous phase containing 1% hydrophilic emulsifier (COG 25) and 1.5% hydrophilic polymer was prepared. Five hydrophilic polymers, tested as stabilizer materials for stabilization of W/O/W emulsion, included alginate, carrageenan, microcrystalline cellulose (MCC), gelatin, and pectin. In the second step of emulsification, the W/O primary emulsion was added into the external aqueous phase with the ratio of 1:1 (w/w). Homogenization of the mixture was conducted at 2,000 rpm for 5 min and then at 2,500 rpm for 10 min to complete the formation of W/O/W emulsion.

Measurement of Emulsion Stability

Aliquots (10 ml) of the emulsions were filled in 10-ml mass cylinders and stored in an oven at 55°C for 7 days. After the phase separation of emulsion occurred, the volume of translucent aqueous phase underneath was recorded. The results were expressed as emulsion stability index (ESI). This gives a range of possible results of 0 to 1; a value of 0 represents poor emulsion stability, a value of 1 represents the best emulsion stability.

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

Structure Observation of Emulsion

The morphology and the droplet size of emulsion were observed by light microscopy (Microlux, Seiler Instrument Co., St. Louis, U.S.A.) at 600× magnification and laser scanning microscopy (LSM 510, Carl Zeiss Inc., Oberkochen, Germany) at 2,500× magnification.

Storage Stability of Formulated Product

Both formulated cells in emulsion and unformulated cells in culture broth were stored at 4°C and 37°C. Cell viability was monitored weekly during 20 weeks at 4°C and 14 weeks at 37°C. The number of viable cells in the samples was assayed by plating the serial dilutions on Luria broth agar (LBA) medium. Cells were incubated at 37°C for 24 h, and the number of visible colonies was then counted. Each sample was assayed in triplicate.

In Vitro Determination of Antagonistic Activity

Antagonistic activities of formulated cells in emulsion and unformulated cells in culture broth against bacterial wilt pathogen *R. solanacearum* were tested *in vitro* by applying them in soil medium. Activity was assessed using the following protocol. Two samples, emulsion and culture broth, were incorporated into soil at 0, 8, 16, and 20 weeks during storage at 4°C. Soil without the samples treatment (incorporation) was used as control. Sample (1 ml) was mixed with 2 ml of sterilized 0.85% NaCl solution, and the mixture was then poured into a test tube containing 3 g of sterilized soil medium. After agitation with a vortex mixer, sample mixed with soil medium was incubated at 30°C for 24 h. Then, 0.5 ml of *R. solanacearum* suspension (10^7 CFU/ml) was inoculated into the above soil medium and incubated at 30°C for 6 days. One gram of soil was used to assay for population densities of pathogen. Serial dilutions were prepared, and 0.1 ml aliquot was spread on *R. solanacearum* selective medium (10 g peptone, 1 g casamino acid, 5 g glucose, 5 mg crystal violet, 50 mg 2.3.5-triphenyl tetrazolium chloride, 20 g agar, 1 l distilled water, pH 7.0). Colonies on agar plates were counted after the incubation at 37°C for 18 h. The pathogen population was represented as CFU/g soil. The entire trial was repeated, and the data presented are means of three trials.

RESULTS AND DISCUSSION

Characterization of Emulsion

Maintaining an emulsion stability requires an emulsifying agent. The emulsifying agent lowers the surface tension of the droplets and forms a barrier to help prevent coalescense of the droplets [18]. Thickening agents such as gums and hydrocolloids are used as natural emulsifying agents. The five hydrophilic polymers were tested to enhance the stability of multiple emulsion. Cellulose showed better emulsion stability than the other types of polymers (Fig. 1).

Normal microscopic examination lacks sufficient resolution and depth of focus to reveal the details of the microstructure of the concentrated emulsion. We, therefore, used a laser scanning microscope under higher magnification to observe the detailed structure and to investigate the entrapment of cells within oil globules. As shown in the micrograph of

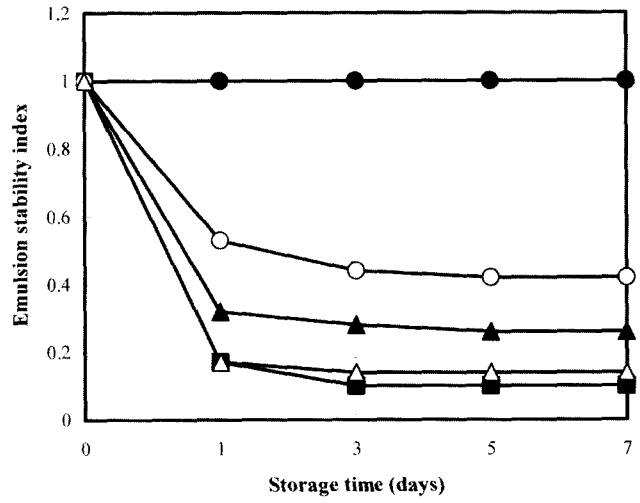

Fig. 1. Comparison of various hydrocolloid gums (stabilizers) for stabilization of W/O/W emulsion during storage at 55°C.
●: Microcrystalline cellulose; ■: Alginate; ▲: Carrageenan; ○: Gelatine; △: Pectin.

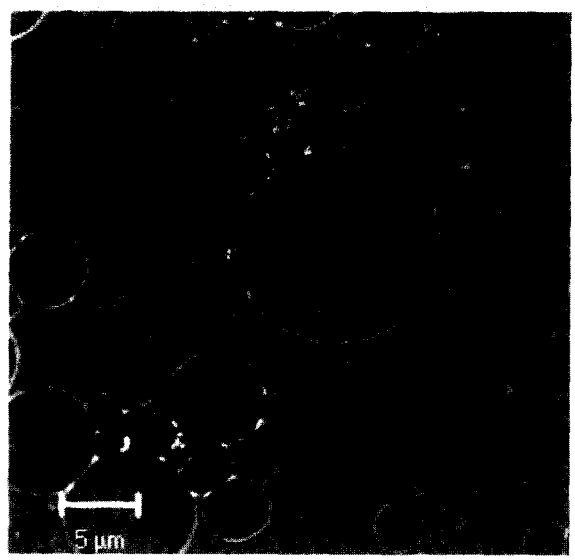

Fig. 2. Photograph of W/O primary emulsion (magnification ×2,500).

W/O emulsion (Fig. 2), the droplets of aqueous medium (culture broth) containing cells were surrounded by the oil phase. The diameter of W/O emulsion globules is about 1–13 μm. Larger-sized droplet of culture broth contained more cells. The diameter of W/O/W emulsion globules ranged from 10 to 100 μm under a light microscope (Fig. 3A). Higher magnification micrograph showed that W/O/W emulsion globules consisted of a large number of small aqueous droplets entrapped within the oil membrane (Fig. 3B). It is noted that they have a structure composed of spherical water domains surrounded by a thin layer of oil. The addition of adjuvant oils involves oil encapsulation technology, therefore the active ingredient is enclosed in a protective shell of oil [1].

Storage Stability

The viability of formulated cells in emulsion and unformulated cells in culture broth during storage at 4°C and 37°C are shown in Fig. 4. At 4°C, the number of viable cells in

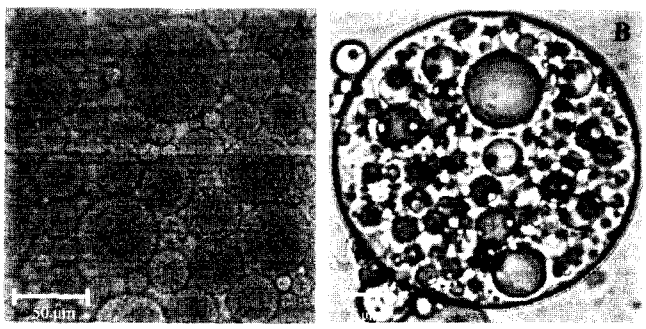

Fig. 3. Photographs of W/O/W multiple emulsion.
(A) Magnification ×600, (B) magnification ×2,500.

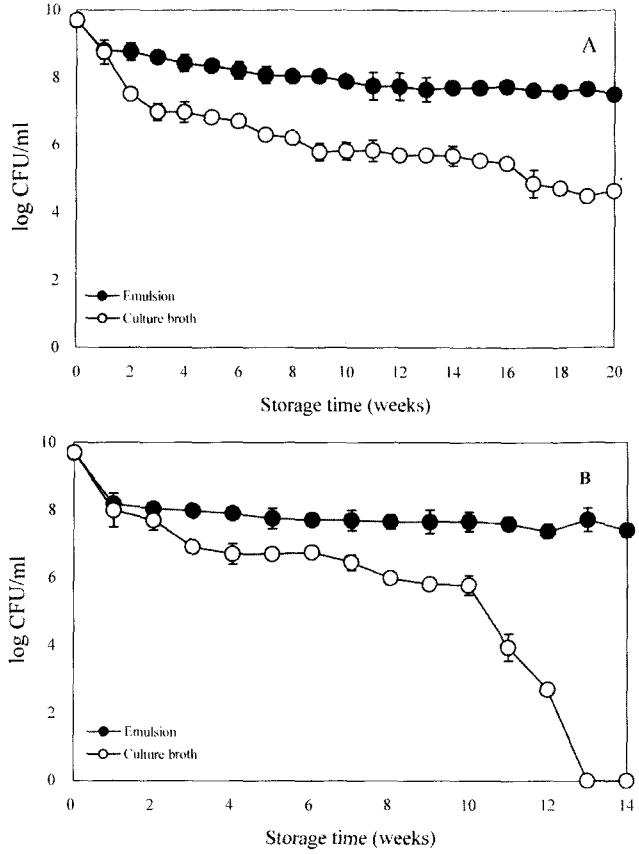

Fig. 4. Viability of *B. gladioli* in emulsion formulation and in unformulated culture broth stored at 4°C for longer than 20 weeks (A) and 37°C for over 14 weeks (B).
Viability was measured at 1-week interval. The vertical bars represent the standard error of the mean.

culture broth decreased from an initial cell count of 5.0×10^9 to 3.2×10^7 CFU/ml after 2 weeks and continued to decline to 4.2×10^4 CFU/ml at the end of storage. On the other hand, the number of viable cells in emulsion decreased from an initial cell count of 4.8×10^9 to 7.8×10^7 CFU/ml after 10 weeks and remained stable throughout the storage periods. At 37°C, the number of viable cells in culture broth decreased from 5.0×10^9 to 6.0×10^5 CFU/ml after 10 weeks. Thereafter, cell numbers sharply declined and viable cells were not detected after 13 weeks of storage, while the number of viable cells in emulsion decreased from 4.8×10^9 to 9.5×10^7 after 3 weeks and remained stable until the end of storage. The reason for these results is not clear, however it may be due to a slower metabolic rate of the cell entrapped within oil in emulsion than that of the cell in culture broth under similar storage condition. It is possible that the limited aeration of the cell surrounded by oil membrane may minimize the metabolic rate of the cell and prevent accumulation of toxic metabolites and depletion of nutrients, consequently prolonging the life span [4].

Table 2. Inhibitory effect of culture broth and emulsion containing *B. gladioli* on growth of *R. solanacearum* in soil medium.

Treatment	Population of *R. solanacearum* (CFU/g soil)			
	Storage period of samples (weeks)			
	0	8	16	20
Control	$(1.0\pm0.5)\times10^{8}$	$(3.8\pm1.0)\times10^{8}$	$(1.3\pm0.1)\times10^{9}$	$(6.8\pm1.8)\times10^{7}$
Culture broth	$(8.9\pm0.4)\times10^{5}$	$(6.5\pm0.8)\times10^{7}$	$(5.1\pm0.5)\times10^{8}$	$(4.3\pm0.3)\times10^{7}$
Emulsion	$(6.4\pm1.0)\times10^{5}$	$(1.3\pm0.1)\times10^{6}$	$(6.3\pm1.9)\times10^{7}$	$(9.9\pm0.5)\times10^{5}$

Each value in the table is mean±standard deviation of three trials.

Most conidia used as potential mycoinsecticide or mycoherbicide have been encapsulated by emulsifiable oil, such as vegetable oil or mineral oil, to protect against the deleterious effect of sunlight [1, 11]. These studies have shown that formulation in oil has an important role to play in the optimization of activity for a pesticide, compared with conventional water-based formulation. Vegetable oil was used in our study to enhance the storage stability of *B. gladioli* strain with shorter shelf life during extended period. Therefore, the use of emulsifiable oil appears to have a great potential for formulation of various BCAs.

In Vitro Determination of Antagonistic Activity

As mentioned before, *B. gladioli* strain is known to effectively inhibit microorganism against growth of *R. solanacearum*. In our preliminary studies, treatment with cell-free emulsion did not differ from untreated control in inhibiting the growth of pathogen (data not shown). At the beginning of samples storage, both formulated cells in emulsion and unformulated cells in culture broth showed great antagonistic activity against *R. solanacearum* pathogen (Table 2). Treatment with freshly prepared emulsion and culture broth resulted in 2.19 and 2.05 log reduction of pathogen in soil, respectively, as compared with the untreated control. However, antagonistic activity of cells in culture broth decreased rapidly in 8 weeks of storage. Population density of pathogen in soil treated with 8-week-old culture broth showed only 0.77 log reduction, as compared with the untreated control. On the other hand, the cells in emulsion were shown to be effective in inhibiting the growth of pathogen even after 20 weeks of storage (Table 2). The activity loss of the cells in culture broth might have been due to a reduction in population of the cells during storage period. These results clearly showed that treatment of soil with a lower population level of antagonist failed to inhibit the growth of pathogen. Vidhyasekaran *et al.* [26] also suggested that the reduction in the efficacy of the unformulated bacterial cell suspension stored for a long time appears to be due to the reduction of populations of the bacterial strains. Hence, effective disease control by soil application with antagonist may be needed to maintain the high population level of the product containing antagonist.

Multiple emulsion of W/O/W type has many potential applications, however, as yet no real commercial product on the market. The main reason is the inherent instability of the preparation. In this study, the use of MCC to serve as a steric stabilizer for outer interface was very helpful in obtaining a multiple emulsion with excellent stability. Also, the formulated form (emulsion) exhibited better storage stability and activity than the unformulated form (culture broth).

In conclusion, the W/O/W multiple emulsion system was shown to be useful as a novel formulation technique of microbial pesticide for agricultural application.

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