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ABSTRACT

Under this USDOE-NETL contract, the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) is being developed as a biocontrol agent for control of the two species of zebra mussels (*Dreissena polymorpha* and *Dreissena bugensis*) that infest water pipes in power plants in North America. Progress was made in the following areas during this reporting period:

- The microcrustacean *Daphnia magna* is an aquatic filter feeder that ingests small suspended particles including bacteria, making it an appropriate organism for non-target tests. Experiments conducted during this reporting period indicate that *Pf*-CL145A is not lethal to this species. This further advances the potential for commercialization of *Pf*-CL145A as *D. magna* is one of the primary species that the U.S. Environmental Protection Agency uses to assess biopesticide safety.
- In experiments conducted during this reporting period, the cost of the fermentation medium needed to produce high yields of toxic *Pf*-CL145A cells was reduced by ca. 88%. This new fermentation medium, in conjunction with a newly revised fermentation protocol, will serve as the basis for future commercial production of large quantities of *Pf*-CL145A at relatively low cost. Thus, this bacterial approach to zebra mussel control has now become more economically competitive with the cost of biocides currently used by power plants.
- In late 2005, we submitted a $100,000 request to the National Science Foundation (NSF) to work collaboratively with a St. Louis company, Particle and Coating Technologies (PCT), to develop a method to dry the bacteria so that loss of toxicity when stored as a commercial product would be minimal. This NSF program is specifically designed to advance the commercialization of biotechnological products by funding joint industry-governmental research collaborations. NSF awarded this grant, and we now have begun to conduct joint research with PCT. This is another encouraging development on the path to commercialization of *Pf*-CL145A.

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EXECUTIVE SUMMARY

Use of the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) represents a potential alternative to the current use of polluting biocides for control of zebra mussel infestations within power plant water pipes. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent so that it will be feasible to use against the two zebra mussel species infesting North American power plants, *Dreissena bugensis* and *D. polymorpha*. During the last six months, research efforts focused on the following activities:

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

Daphnids are aquatic filter-feeding microcrustaceans that normally ingest suspended particles such as bacteria, making them appropriate candidates to include in our safety trials. Herein we report our latest non-target trials evaluating the acute lethality of *Pf*-CL145A to the *Daphnia magna*.

Non-target trials to date with *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) have been very encouraging. In tests using dead *Pf*-CL145A cells, no mortality has been observed among any of the following non-target organisms: the ciliate *Colpidium colpoda*, 7 bivalve species (*Mytilus edulis, Pyganodon grandis, Lasmigona compressa, Strophirus undalatus, Lampsilis radiata, Pyganodon cataracta, Elliptio complanata)*, and 3 fish species (*Pimephales promelas*, *Salmo trutta*, and *Lepomis macrochirus*).

Test results indicated that *Pf*-CL145A cells were not lethal to daphnids when treated at 200 ppm for 48 hr. Overall mean daphnid mortality (±SD) among the 4 tests conducted was 5.0(±5.8)% in treated dishes and 6.3(±7.5)% in untreated controls. In contrast, concurrent tests against zebra mussels at 200 ppm for 24 hr resulted in an overall mean mortality (±SD) of 92.7(±2.4)% (control mortality 2.2(±1.7)%). These results are further evidence that this strain of *P. fluorescens* has unusual host specificity and suggest its use as a zebra mussel biocontrol agent will pose relatively low environmental risk.

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING THE INEXPENSIVE COMMERCIAL MEDIUM.

In the previous reporting period, we described the development of an inexpensive medium with which medium cost was reduced using shake-flask cultures. In the current reporting period, a fermentation protocol was developed in which toxic cells of *Pf*-CL145A could be produced using a similar inexpensive commercial medium in 0.5-L fermentors. Based on a series of 0.5-L fermentations, we estimate that the cost to produce 1 Kg (dry weight) of *Pf*-CL145A would cost ca. $72 in the new commercial medium (referred to as FM4) compared to ca. $587 if the previous medium (FM2) was used, a cost reduction of ca. 88%. Future large scale fermentations for commercial production of a *Pf*-CL145A-based biocontrol product will be based on this medium and protocol.

3. GRANT AWARDED BY NATIONAL SCIENCE FOUNDATION.

Cell toxicity stabilization is a critically important aspect to successfully developing a commercial product. When the bacterial cells are grown up and harvested, they must be processed in a manner that maintains their toxicity to zebra mussels. In late 2005, we submitted a $100,000 request to the National Science Foundation (NSF) to work collaboratively with a St. Louis company, Particle and Coating Technologies (PCT), to develop a method to dry the bacteria so that loss of toxicity when stored as a commercial product would be minimal. This NSF program is specifically designed to advance the commercialization of biotechnological products by funding joint industry-governmental research collaborations. NSF awarded this grant, and we now have begun to conduct joint research with PCT. This is another encouraging development on the path to commercialization of *Pf*-CL145A.

INTRODUCTION

Coal-fired power plants within North America need an effective, economical, and non-polluting technique for managing infestations of zebra mussels within their facilities. Due to a lack of options, many facilities have relied on the use of broad-spectrum, chemical biocides for control of these freshwater mussels. However, biocide treatments, such as continuous chlorination for three weeks, are widely regarded as environmentally unacceptable because they can result in the formation of potentially carcinogenic substances. Use of the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A)

represents a potential alternative to the use of polluting biocide treatments and is the leading candidate in the world for the biological control of these macrofouling mussels. The purpose of this DOE-NETL project is to accelerate research toward commercialization of this biocontrol agent so that it will be feasible to use against the two zebra mussel species infesting North American power plants, *Dreissena bugensis* and *D. polymorpha*. During the last six months, progress was made in the following areas:

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

In terms of environmental safety, non-target trials to date with *Pseudomonas fluorescens* strain CL145A have been very encouraging. In tests using dead *Pf*-CL145A cells, no mortality has been observed among any of the following non-target organisms: the ciliate *Colpidium colpoda*, 7 bivalve species (*Mytilus edulis, Pyganodon grandis, Lasmigona compressa, Strophirus undalatus, Lampsilis radiata, Pyganodon cataracta, Elliptio complanata)*, and 3 fish species (*Pimephales promelas*, *Salmo trutta*, and *Lepomis macrochirus*). Despite these results, it would be unreasonable to think that this strain will prove so selective as to only affect zebra mussels. For this reason, further non-target tests are ongoing to satisfy U.S. Environmental Protection Agency (USEPA) biopesticide registration requirements. Daphnids are aquatic filter-feeders that normally ingest suspended particles such as bacteria, making them appropriate candidates for such trials Therefore, tests were conducted with zebra mussels and the non-target organism *Daphnia magna* at a target treatment concentration of 200 ppm to determine if high zebra mussel mortality can be attained while receiving little or no *Daphnia* mortality.

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING THE INEXPENSIVE COMMERCIAL MEDIUM.

In the previous reporting period, we described the development of an inexpensive medium with which medium cost was reduced by 89%. In these latter experiments, the bacteria were cultured in shake-flask cultures. However, for commercial-scale production of *Pf*-CL145A, culturing in shake-flasks is not practical, so the bacteria must be cultured in fermentors where various parameters such as pH, oxygen, and agitation can be carefully monitored and controlled over the life of the culture. The modification of any one or combination of parameters can greatly affect the production of toxic cells. Therefore, in the current reporting period, a protocol was developed in which toxic cells of *Pf*-CL145A could be produced using the inexpensive commercial medium in 0.5-L fermentors. The development of this detailed fermentation protocol will allow us to scale-up fermentations for future production requirements at a fraction of the cost compared to using more expensive media.

3. GRANT AWARDED BY NATIONAL SCIENCE FOUNDATION.

Cell toxicity stabilization is a critically important aspect to successfully developing a commercial product. When the bacterial cells are grown up and harvested, they must be processed in a manner that maintains their toxicity to zebra mussels. In late 2005, we submitted a $100,000 request to the National Science Foundation (NSF) to work collaboratively with a St. Louis company, Particle and Coating Technologies (PCT), to develop a method to dry the bacteria so that loss of toxicity when stored as a commercial product would be minimal. This NSF program is specifically designed to advance the commercialization of biotechnological products by funding joint industry-governmental research collaborations. NSF awarded this grant, and we now have begun to conduct joint research with PCT. This is another encouraging development on the path to commercialization of *Pf*-CL145A.

EXPERIMENTAL

The following is an overview of the materials and methods used in above-mentioned research projects:

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

- Preparation of bacteria: Frozen CF (Cell Fraction) that had been irradiated to kill the cells was used for these tests to insure that both the target and non-target organisms were treated with dead cells only. The bacteria were produced in 100-L fermentation units at the Center for Biocatalysis and Bioprocessing at the University of Iowa, Iowa City, Iowa and irradiated at E-Beam Services, Inc. in Cranbury, New Jersey.

- Source of *Daphnia magna*: The *Daphnia magna* (Fig. 1) stock used in these tests was originally obtained from the NYS Department of Environmental Conservation Bureau of Environmental Protection (Avon, New York).

Fig. 1. *Daphnia magna*, a filter feeding microcrustacean.

- Maintenance of *Daphnia* cultures prior to testing: The United States Environmental Protection Agency (2002) document on methods to measure acute toxicity was used as a general guide for the following *Daphnia* maintenance and testing. *Daphnia* were cultured in polystyrene cups containing 20 ml of aerated *Daphnia* HW (hard water) set up in a rack that held 50 cups total (Fig. 2). The colony was maintained in a 21-23°C room on a 16 hr light/8 hr dark light cycle at a light intensity of 50-100 foot candles. Each day the adult *Daphnia* were transferred to cups containing fresh HW and fed 0.1 ml YCT (yeast, Cerophyll®, and digested trout starter food) and 0.1 ml of an algal concentrate of *Selenastrum capricornutum* (Fig. 3). The neonates that were produced overnight were discarded, unless they were to be used for testing that day. New cups were started every three weeks with ≤24 hr old neonates. In addition, *Daphnia* were cultured in bottles (50 *Daphnia*/bottle) containing 2 L of aged, unchlorinated tap water to serve as back-up cultures in the event the cups failed. Water was changed every Monday, Wednesday, and Friday, and each bottle was fed 1.5 ml each of YCT and algal concentrate per day. Neonates produced were discarded during each water change. New bottles were started every three weeks using 50, 24-48 hr old neonates.

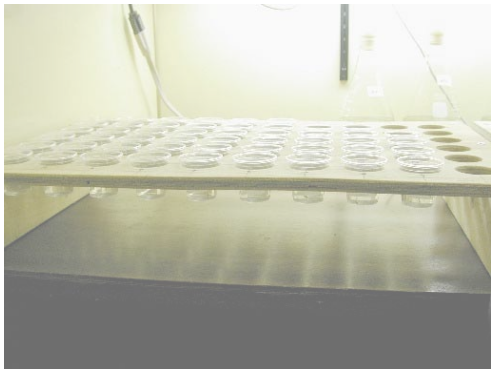

Fig. 2. *Daphnia* cultures in cups.

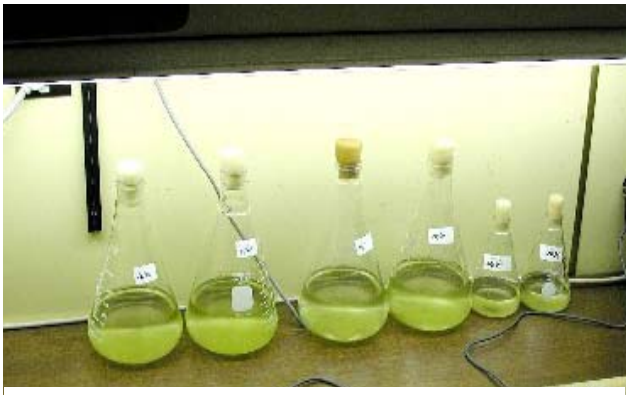

Fig. 3. Algal food and stock cultures used to feed *Daphnia* cultures.

- *Daphnia* treatment: Only neonates ≤24 hr old were used for testing in cups. The day preceding the test, adult *Daphnia* were transferred to new cups containing fresh HW and fed. The next day (the day of the test) the adults were again transferred to fresh cups, leaving behind the neonates produced overnight. Five neonates were transferred, 1 at a time, to each labeled Petri dish containing 25 ml of *Daphnia* hard water pre-treated at 200 ppm (Fig. 4). Neonates were fed at

the start of the test and again at 24 hr. The dishes were placed in a constant temperature incubator set at 23°C for the duration of the treatment period (Fig. 5). After the 48 hr exposure period, the treated *Daphnia* were transferred to new Petri dishes containing 25 ml of fresh, oxygenated hard water and fed 0.1 ml YCT and 0.1 ml algal concentrate. *Daphnia* were held for an additional 8 days, during which time on a daily basis the water was changed, daphnids were fed, and mortality was scored.

Fig. 4. Treating Petri dishes containing *Daphnia* neonates with *Pf*-CL145A.

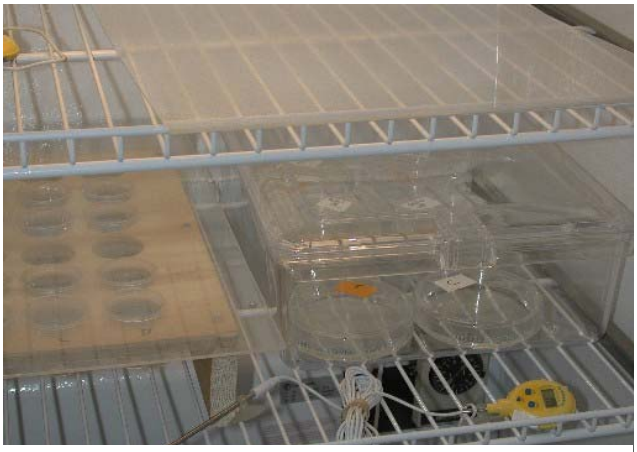

Fig. 5. Treated and control Petri dishes with *Daphnia* neonates in 23°C incubator.

- Preparation of mussels: Zebra mussels were collected from the Mohawk River near Crescent (NY), brought back to the lab, sieved and kept at 7ºC in filtered, aerated, unchlorinated tap water until testing began. Approximately 1 week before the test, mussels were removed from the cooler (7ºC) and acclimated to 23ºC.
- Standard laboratory bioassays of mussels with CF: The day before treatment, mussels were picked and placed in jars containing ca. 100 ml of aerated HW and then allowed to attach overnight. The morning of treatment, unattached mussels were removed and replaced with attached mussels from an extra dish. At least 1 hr before treatment, the testing containers were filled with 495 ml of fresh hard water, set up with aeration, and labeled. Mussels were exposed for the treatment period (24 hr). The treated hard water was then poured off, and the mussels were transferred to clean plastic dishes with fresh, oxygenated hard water and examined for mortality. Mortality was recorded and the mussels held in the dishes for an additional 9 days, with water being changed and mortality being scored each day.

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING THE INEXPENSIVE COMMERCIAL MEDIUM:

- Shaken seed cultures: 250-mL Erlenmeyer flasks containing seed culture medium (bTSB or FM4) were inoculated with 0.4 mL of stock culture and shaken at 200 rpm at 26±1ºC for 24 hr.
- Shaken flask cultures: Replicate flasks containing 35 mL of the experimental culture medium were inoculated at a 1% (v:v) concentration with the 24-hr seed culture. Flasks were shaken at 200 rpm at 26±1ºC for 24 hr.
- Fermentations: Experimental fermentations were conducted using six 0.5-L (working volume) fermentors (Appropriate Technical Resources, Inc.) (Fig. 6). Medium components and fermentation parameters were modified in duplicate fermentors to aid in the development of the new fermentation medium (FM4) and protocol. Individual 0.5-L fermentors were inoculated at a 1% (v:v) concentration with the 24-hr seed culture. Sub-samples from the cultures were removed over the culture period from 0 to 24 hr to determine the optimal harvest time.
- Biomass production: Biomass production in shake-flask cultures was determined from optical density readings ($A_{660\ nm}$; Genesys 20 Spectrophotometer) of the 24-hr final whole culture (FWC).

- Production of cell fraction (CF): The final whole culture (FWC) from each culture was centrifuged and the pellets were combined to produce a common pellet. Cell pellets were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \cdot 6H_2O$ in deionized water).
- Preparation of CF: Mean dry bacterial cell mass/mL for each CF was calculated from two 1.0 mL desiccated subsamples using a Denver Instruments balance. The amount of inoculum needed to treat at the targeted concentration was based on the mean dry bacterial cell mass/mL.
- Preparation of mussels: Zebra mussels were collected from various locations, brought to the testing site, sieved, and stored until testing began. Mussels were collected from the Mohawk River (Crescent, NY), Hedges Lake (Cambridge, NY), and the Champlain Canal (Fort Edward, NY).
- Standard laboratory bioassays of mussels with CF: The day before treatment, mussels were picked, placed into testing containers (either micro-chambers or testing jars) containing 5 or 100 mL of aerated hard water (Peltier and Weber, 1985), respectively, and allowed to attach overnight. The morning of treatment, unattached mussels were replaced with attached mussels from an extra dish. At least one hour before treatment, the testing containers were filled with fresh aerated hard water (10 mL for micro-chambers, 500 mL for testing jars), set up with aeration, and labeled (Fig. 7). Mussels were exposed for the treatment period (24 hr). Then the fluid was poured off and mussels were transferred into clean plastic dishes with fresh, oxygenated hard water to be examined for mortality. Mussels were held in the dishes for an additional 6 (micro-chambers) or 9 (testing jars) days, with the water being changed and mortality being scored each day. All binomial mortality data were analyzed following angular transformation (Sokal and Rohlf, 1995).

Fig. 6. 0.5-L Sixfors fermentors used to develop the *Pf*-CL145A fermentation protocol with inexpensive medium.

Fig. 7. Three replicate micro-chambers containing zebra mussels with aeration.

RESULTS

This section gives an overview of the progress made during the six-month reporting period in both planning and conducting of the research experiments:

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

At the end of the 48 hr exposure period, no *Daphnia magna* mortality was observed in any of the tests conducted (Table 1). By day 10, the mean *Daphnia* mortality in treated and control dishes was 5.0 (±5.8)% and 6.3 (±7.5%), respectively. In contrast, mean zebra mussel morality in treated and control jars for all tests conducted was 92.7 (±2.4)% and 2.2 (±1.7)%, respectively. During the treatment period dissolved oxygen levels remained at or above the United States Environmental Protection Agency (2002) suggested minimum of 5.0 ppm.

Table 1. Summary of the tests conducted with *Daphnia magna* and zebra mussels.

	Laboratory Test #	Daphnid 48 hr mean % mortality (200 ppm)	Daphnid 10-day mean % mortality (200 ppm for 48 hr)	Daphnid 10-day mean % mortality (untreated controls)	Mussel 10-day mean % mortality (200 ppm for 24 hr)	Mussel 10-day mean % mortality (untreated controls)
	2005-0030	0.0	0.0	0.0	no mussel tests done	no mussel tests done
	2005-0033	0.0	10.0	15.0	95.3	0.7
	2005-0045	0.0	0.0	0.0	90.7	2.0
	2005-0046	0.0	10.0	10.0	92.0	4.0
Mean		**0.0**	**5.0**	**6.3**	**92.7**	**2.2**
SD		**0.0**	**5.8**	**7.5**	**2.4**	**1.7**

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING THE INEXPENSIVE COMMERCIAL MEDIUM.

A detailed fermentation protocol was developed through a series of fermentations in the 0.5-L Sixfors fermentors (Fig. 6) by comparing the biomass production and toxicity of fermentations of *Pf*-CL145A in the new commercial medium (FM4) to values from the previously developed fermentation medium (FM2). The relative cost of FM4 was 0.17, which corresponds to an 82.8% decrease in medium component cost compared to the previously developed medium (Fig. 8, Table 2). Cells of *Pf*-CL145A harvested from fermentations with FM4 were slightly less toxic than cells harvested from FM2 (average relative toxicity value = 0.92, Fig. 8). However, at the time of harvest of the most toxic cells from each type of medium, fermentations in FM4 produced over 40% more biomass of cells (Fig. 8), thus further reducing the overall cost of the *Pf*-CL145A cell-based product. Considering the relative biomass produced, the estimated cost of media to produce 1 Kg (dry weight) of cells using the new commercial-grade medium, FM4, is $72.09, compared to $587.28 using the previous medium, FM2.

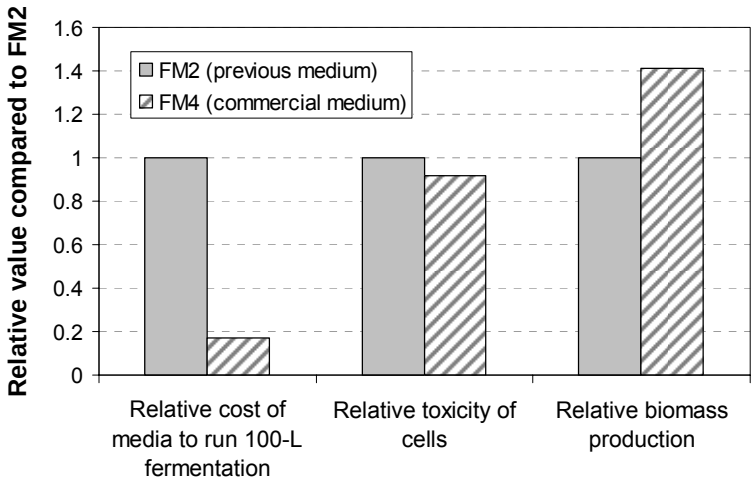

Fig. 8. Comparison of relative cost, toxicity and biomass production of the cheaper commercial medium (FM4) and the previous fermentation medium (FM2).

Table 2. Estimated cost of two fermentation media: the previously developed medium, FM2 and the new commercial medium, FM4. Total cost of media for a 100-L fermentation includes the cost of all media; seed culture and fermentor culture.

Cost comparison for 100-L fermentation run			
Medium	**$ 100-L fermentation medium**	**$ 1-L seed culture**	**TOTAL cost of media to inoculate a 100-L run**
FM2	$232.43	$2.48	$234.91
FM4	$40.00	$0.37	$40.37
		% REDUCTION =	**82.8%**

DISCUSSION

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

While zebra mussel kill was lower than expected when treated at a concentration of ca. 200 ppm, the results of these initial tests with the non-target organism, *Daphnia magna* are very encouraging and provide further evidence to support the environmental safety of *Pf*-CL145A as a bio-control agent. The ideal non-target test is one in which the inoculum being used causes very high kill (e.g., >95%) against the target pest. The slightly lower zebra mussel kill seen in this test was almost certainly related to the fact that the inoculum has been over-irradiated at E-Beam, i.e., the cell kill of 99.9999999% was far greater than desired. Additional bioassays indicated that this irradiation treatment had considerably reduced the toxicity of the cells, i.e., the same dosage (50 ppm/24 hr) that gave 97% kill with non-irradiated cells gave only 77% kill following this irradiation. During the treatment period, *Daphnia* and zebra mussels were exposed to *Pf*-CL145A at a target concentration of 200 ppm as this dosage level has been demonstrated to produce over 95% zebra mussel kill. This treatment concentration however, does reduce dissolved oxygen levels during the first 24 hr to the minimum (5.0 ppm) suggested by EPA. As research continues on improving the toxicity of the bacterial cells, fewer cells will be needed (e.g., <50 ppm) to achieve the same or higher zebra mussel kill. It is anticipated that future trials with *Daphnia magna* using more toxic cells at lower treatment concentrations will also result in little or no mortality of this non-target species.

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING INEXPENSIVE COMMERCIAL MEDIUM.

Although the toxicity of *Pf*-CL145A cells harvested from fermentations using FM4 appeared to be slightly lower (ca. 8%) than cells from the previously developed medium, FM2, the overall cost reduction (ca. 83%) and biomass production increase (ca. 40%) were significant improvements. If our plans for genetically increasing the toxin production of each cell of *Pf*-CL145A are successful, it should more than compensate for this slightly lower toxicity. Progress toward the genetic improvement of *Pf*-CL145A is currently underway with the submission its genomic DNA to 454 Life Sciences, Inc. for sequencing. The inexpensive medium will likely remain applicable for commercial production of large quantities of highly toxic *Pf*-CL145A product at a fraction of the cost compared to previously developed media.

CONCLUSIONS

1. NON-TARGET TRIALS WITH *DAPHNIA MAGNA*.

Research to date indicates that the bacteria, *Pf*-CL145A is non-toxic to *Daphnia magna* at a treatment concentration as high as ca, 200 ppm for 48 hr. As *Daphnia* are filter feeders, ingesting suspended particles including bacteria, this is very encouraging and provides further evidence as to the environmental safety of *Pf*-CL145A as a biocontrol agent. As research continues on improving toxin production per cell and thus lowering the quantity of bacteria required to achieve high zebra mussel

mortality (e.g. >95%), it is expected that future non-target trials with *D. magna* will result in little or no *Daphnia* mortality.

2. DEVELOPMENT OF FERMENTATION PROTOCOL FOR *PF*-CL145A USING THE INEXPENSIVE COMMERCIAL MEDIUM.

A fermentation protocol was developed using an inexpensive medium, FM4, in which toxic cells of *Pf*-CL145A were produced in 0.5-L fermentors. We estimate that the cost to produce 1 Kg (dry weight) of *Pf*-CL145A would cost ca. $72 in the new commercial medium (FM4) in fermentors compared to ca. $587 if the previous medium was used (FM2), a cost reduction of ca. 88%. Future large scale fermentations for commercial production of a *Pf*-CL145A-based biocontrol product will be based on this medium and protocol.

3. GRANT AWARDED BY NATIONAL SCIENCE FOUNDATION.

The NSF grant that was recently awarded to develop a process that will stabilize cell toxicity will accelerate the path to commercialization of this bacterial control agent.

REFERENCES

Peltier, W. H. and Weber, C. I. 1985. Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Third edition. U. S. Environmental Protection Agency Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio. 216 pp.

Sokal, R. R. and Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. Third edition. W. H. Reeman and Company, New York. 887 pp.

United States Environmental Protection Agency. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth edition. EPA-821-R-02-013. U. S. Environmental Protection Agency, Office of Water (4303T), Washington, D.C. 335 pp. Available at http://www.epa.gov/OST/WET/disk3/