U.S. DEPARTMENT OF AGRICULTURE

SMALL BUSINESS INNOVATION RESEARCH

PHASE I AND PHASE II

PROJECT SUMMARY*

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Program Office	Solicitation No.	Proposal No.	Topic No.

TO BE COMPLETED BY PROPOSER

Name and Address of Firm	Name and Title of Principal Investigator(s)
Protein Solutions, Inc. 6009 Highland Dr. Salt Lake City, UT 84121	J.D. Andrade President and Chief Scientific Offic

Title of Project (80-character maximum)

Aquaculture of Marine Phytoplankton in Enclosed Environments

Technical Abstract (200-word limit)

Certain Marine phytoplankton can be maintained in culture in completely sealed envi for extended periods of time. The ability of phytoplankton to grow in such sealed environ a wide range of possible applications, including the development of living specimens for s education and as detectors and sensors for various agents. Laboratory and large scale cul phytoplankton are open systems. There is virtually no literature on the growth of these c sealed or closed environments.

Bioluminescent dinoflagellates are of particular interest as the light emission pro indicator of the numbers and viability of the cultures.

We propose to perform fundamental studies on the growth, longevity, and stability c cent phytoplankton cultures in totally materially sealed and partially gas permeable envir goal is to understand the photosynthesis/respiration ratios, and the effect of pH, PO_2, P O_2 and CO_2 concentrations in the gas phase. These parameters will be studied as a functio temperature, lighting conditions, culture medium, and surface to volume ratios. These st have wide applicability to the development of sealed phytoplankton cultures for education as food for marine organisms, as a means to facilitate the transport of stocks from site t as a simple means of producing phytoplankton for biotechnological studies.

Anticipated Results/Potential Commercial Applications of Research (100-word limit)

Materials and Methods for producing phytoplankton for

1) the pharmaceutical and biotechnology industries;
2) the home hobby aquarium market;
3) the display aquarium market;
4) science education;
5) as detectors and sensors for toxic agents
6) food for other aquaculture applications

Keywords to Identify Technology/Research Thrust/Commercial Application (8-word maximum)

Phytoplankton, Bioluminescence, Photosynthesis, Respiration, Aquaculture, Ecosyste Mesocosm, Education.

*The Project Summary must be suitable for publication by USDA in the event of an award. *Do not i proprietary information on this page.*

FORM CSRS-668 (7/87)

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C. TECHNICAL CONTENT

1. Identification and Significance of the Opportunity:

There is rapidly growing public interest in aquaculture as a vehicle for enhancing food production, as sources of unique drugs and biochemical agents, as model systems for the study of toxins and toxin defence processes, as means to help treat wastes and deal with environmental problems, and to facilitate the development of closed and even remote life support systems (1-14).

Protein Solutions Inc. initiated work several years ago on the development of bioluminescent phytoplankton, single cell non-toxic dinoflagellates, for science education purposes. Our original rationale was that the beautiful and mysterious bioluminescence generated by such cultures would stimulate students, their teachers, and their parents to become interested in probing a wide range of scientific subjects. This has proven to be the case, and bioluminescent phytoplankton are now used by the Center for Integrated Science Education at the University of Utah for a wide range of workshops for inservice teachers and for the development of unique, integrated science courses on the University campus.

This activity has led to two product development efforts. One, "Night Life", is a science education kit for junior high and high school students utilizing pyrocystis lunula and conventional tissue culture components. During the development of this product, we learned that this particular organism, and probably other robust dinoflagellates, can be maintained in sealed environments. This was a bit surprising because common wisdom suggests that these organisms are actually quite delicate and difficult to culture (17, 33, 34). In using cultures of these organisms to demonstrate the process of bioluminescence and protozoan biology to the general public, teachers, and students, we had to expose the organisms to less than ideal environments (22-24). In some cases they had to be shipped and kept in the dark for extended periods of time. In other cases they had to be completely sealed to eliminate possibility of spillage. In some cases they were exposed to adverse temperatures.

Based on the scientific literature and on our discussions with scientists knowledgeable in these organisms, we were very concerned that they would not survive such traumatic conditions. At the same time we were studying the phenomena of materially closed ecosystems--totally sealed systems in which life manages to co-exist in a microcosm or miniature ecosystem (27). We began to consider the possibility that perhaps the dinoflagellates could also develop their own micro ecosystem.

We now realize that most investigators greatly underestimated the durability and tolerability of certain of these organisms. We learned that cultures of certain dinoflagellates could exist for 6 months or more in a completely sealed environment. The cultures develop a balance between photosynthesis and respiration, which apparently allows them to reach a partial steady state and to survive in small volume sealed cultures for 6 months or more.

We also know that there ar a variety of marine bacteria which co-culture with the organism of interest, thus there may be some symbiosis or at least balance among several organisms in the miniature ecosystem.

The fact that organisms that were considered frail, delicate, and difficult to culture are actually hardy, tolerant, and capable of survival in non-ideal situations and environments provides a unique set of opportunities.

We have now sealed small, low density cultures of the marine dinoflagellate pyrocystis lunula in low density polyethylene bags. This material provides reasonable rates of oxygen and carbon dioxide transport while minimizing water loss from such a closed culture. Although the culture appears to be "closed", it is actually exchanging O_2, CO_2 and water with its ambient environment, albeit the exchange is slower than in a normal culture vessel with an open air/water interface. The small volume cultures last for four to six months, depending on lighting conditions, external temperature, and handling environments. These are small 30 to 40 ml volume cultures. They are being used in a prototype science awareness/education product tentatively called "Galaxsea". This product is designed to introduce the phenomenon of bioluminescence, marine protozoa, and other topics to the general public.

We feel that there is a great potential for the development of larger volume, more optimized cultures enclosed in gas permeable containers. That is the basis of this SBIR application. The availability of larger volume cultures of single-celled algae would be of significant interest for the following applications:

1) as food sources and/or supplements for marine aquaculture (9, 10, 37);
2) as controlled and isolated cultures for basic biological research;
3) as controlled, isolated cultures for the biotechnological and pharmaceutical industry (11, 12);
4) as safe, isolated cultures for individuals studying these organisms;
5) for the production of toxins and other hazardous products; and
6) as means to prepare and deliver such organisms to other environments, including exhibition aquaria, home aquaria, research laboratories, and schools;

We propose to examine the various chemical and physical variables involved in optimizing the culture of marine microalgae in sealed, but gas permeable, environments.

2. Background and Rationale:

Protein Solutions, Inc., and its chief scientific officer and the PI of this proposal, J.D. Andrade, have been working with bioluminescent microorganisms for about four years, the last two years involved in developing the Night Life and Galaxsea pre-products previously noted. We have had extensive interaction with experts in the field, including those knowledgeable about dinoflagellates and other bioluminescent organisms. Our staff has attended the workshop on marine phytoplankton culture and techniques at the Provasoli Guillard Center for the Culture of Marine Phytoplankton in West Boothbay Harbor, Maine.

We have considerable experience with the culture of bioluminescent dinoflagellates in non-traditional media, including artificial sea waters (24), and have had some limited experience on their culture in polyethylene bags (23). Dinoflagellates are normally considered to be delicate organisms which are difficult to culture and maintain (15-17). They also have a reputation of being toxic (15, 16). There are thousands of different dinoflagellates, some produce toxins, many others do not. Many are non-bioluminescent--others are. Many are fragile--some are hardy. After thorough consideration of the scientific literature and discussions with many experts on

dinoflagellates and bioluminescence (15-17, 21, 25, 33, 34), we selected two very hardy, nontoxic, bioluminescent dinoflagellates, which can be maintained in totally sealed environments for up to 6 months. All they require is eight to ten hours/day of light and moderate temperatures.

We originally selected dinoflagellates for our studies because they are the only marine protozoa which are bioluminescent. Although our interests were in the bioluminescence of dinoflagellates, as we have learned more and more about this unique class of organisms, we have become fascinated with their varied characteristics, their distribution throughout the globe, their unique properties and attributes, including bioluminescence, the production of toxins by some species, and their potential as a rich store of new and novel biochemical and possibly pharmaceutical agents (14-17).

There is a growing interest in dinoflagellates--and phytoplankton in general. The recent report describing a new and novel dinoflagellate responsible for East coast fish kills has stimulated much interest (40). The growing occurrences of red tides and algalblooms in many parts of the world is leading to greater and greater scientific and commercial interest in these organisms. The ability to culture and maintain various dinoflagellates in convenient, practical, safe, sealed containers, will be of growing interest to the research and commercial aquaculture community, as well as to the other constituencies noted above.

3. Relation to Future R and D:

It is clear by now that Protein Solutions, Inc. is committed to understanding and developing marine phytoplankton for a variety of purposes. Although our major, immediate product interests are in science education products, it is clear that the technology which the company is developing will be of interest to many other firms in the areas of aquaculture, biotechnology, and pharmaceuticals. We expect that this Phase I SBIR will demonstrate feasibility for larger volume cultures of a wider range of organisms, and will lead to a phase II effort, followed by contract research and development activities with those industries which need access to the unique science and technology which we are developing.

4. Phase I Technical Objectives:

The project has the following specific objectives:

1) Perform a thorough analysis and evaluation of the various types of transparent polymer films suitable to the culture of dinoflagellates in sealed bags. Criteria for film evaluation and selection include: transport of O_2 and CO_2 in water; transparency; mechanical strength; ease of sealing; surface properties of the film, particularly with respect to cell adhesion; and costs and availability.

2) The role of light intensity, light-dark cycle, and temperature on the culture of organisms in the sealed containers.

3) The composition of the culture medium. In order to optimize the longevity of the cultures, changes in the type and amount of nitrogen and phosphorous sources, and the initial pH, will be evaluated.

4) Limited measurements of CO_2, O_2, and pH concentrations in the containers will be performed.

5) An analysis of bag surface area, internal gas volume, and internal liquid volume will be performed, and the surface to gas volume to liquid volume ratios will be optimized for various culture criteria: cell density, longevity, and bioluminescence intensity.

6) Given sufficient time and resources, the above studies will be extended to other photosynthetic dinoflagellates.

5. Phase I Work Plan:

Objective One: Polymer Materials:

Preliminary analysis by Mr. K. Foote of highly gas permeable polymer films for algae aquaculture applications (22) led to the selection of low density polyethylene for our initial studies (Table 1)

Table 1: Best candidates for enclosing membrane material (from Ref. 22).

Membrane Material	Gas Permeability (mol*cm)/(cm²*sec*atm)*10⁻¹²⑧ CO_2	Gas Permeability (mol*cm)/(cm²*sec*atm)*10⁻¹²⑧ O_2	Rate of Water Vapor Transport (g*mil)/(100in²*24hr) at 37.8 °C
Silicone rubber (Silastic)	290	140	170
Low density polyethylene	5.49	1.02	1.3
Ethylene-vinyl acetate copolymer	12.2	1.71	2.5
Fluorinated ethylene propylene copolymer (FEP TEFLON)	3.40	1.52	0.4

Data for all but silicone rubber taken from Modern Plastics Encyclopedia, Vol. 64, 1988, pp 553-557.

⑧ These units for permeability may be converted to the commonly reported units (cc*mil)/(100in²*24hr*atm) by multiplying by 2.033 x 10^{-15}.

Basically one desires maximum CO_2 and O_2 permeability with minimum water vapor transport. The polymer with the highest gas permeability is silicone rubber, but that also has a very high rate of water vapor transport. Very preliminary studies of cultures in silicone rubber bags showed a very rapid water loss, leading to increased salinity of the cultures to the point where cell death occurred.

Low density polyethylene was selected because of the optimum combination of properties, because it is inexpensive, and because it is very easy to heat seal. Most of our experience to date has been with four mil thick polyethylene. We will examine a range of thicknesses and the trade off between thickness, mechanical properties, and gas transfer.

There are many types of low density polyethylene. We have not evaluated the various sources, various densities, crystallinities, and molecular weights. We also have not evaluated ethylene vinyl acetate copolymer, which is very promising and has a different ratio of CO_2 to O_2 permeability, which would be very interesting in terms of fundamental studies of photosynthesis and respiration ratios.

Probably the best material is FEP Teflon. CO_2 and O_2 permeabilities are comparable to polyethylene, with water vapor transport three times less. Unfortunately FEP Teflon is extremely expensive for this application, although it has been used, and is marketed as a cell-culture bag for small volume research applications (41).

We propose to perform limited studies with FEP Teflon and silicone rubber, and much more extensive studies with various grades and types of low density polyethylene and ethylene vinyl acetate copolymer. We will also perform limited studies with polystyrene, polymethylmethacralate, and polycarbonate. These polymers have extremely low gas permeability and water vapor transport rates, and can be considered materially closed containers if properly sealed. Polycarbonate is commonly used for phytoplankton culture (16, 17). These materials will serve essentially as controls for the approximately zero gas transfer and water vapor transfer rate studies.

Objective Two: Role of Light Intensity:

Our studies to date on light intensity have been quite qualitative, ranging from normal room lighting to more intense and controlled laboratory lighting--100 micro Einsteins/meter²/sec. This is equivalent to two 40 watt fluorescent bulbs at about 1 ft. from the culture container. This is the typical intensity used in most culture laboratories working with photosynthetic microalgae (16, 17). Normal light cycles are 12 hours on and 12 hours off, which establishes typical circadian rhythm. Normal culture temperature is about 16° C.

Light intensities in the range of 10 to 200 micro Einsteins/m²/second, temperatures in the range of 5 to 40° C., and the full range of light-dark cycles will be evaluated. Evaluation will consist of cell density in the bag, which will be measured by sampling and direct counting and indirectly measured by bioluminescence intensity and by chlorophyll absorption.

Objective Three: Media optimization:

Our preliminary studies indicate that a normal Guillard f/2 medium (17), but with the initial pH adjusted to about 7.5, will shift to an increased alkalinity after several months, plateauing at a pH of about 8.5 and maintaining that pH for several additional months. These results come from preliminary and quite qualitative studies. Modification of the initial pH of the medium and modification of the buffer type and capacity may have a significant effect on the pH changes during the life of the culture.

The typical media utilize nitrate and phosphate as the sources of nitrogen and phosphorous, respectively. Nitrate uptake is apparently balanced by OH production and increased alkalinity. This effect could be minimized by using ammonia as a nitrogen source, which leads to H+ generation, or perhaps even better by using the uncharged nitrogen compound, urea, in which pH changes are apparently minimal (18, 31, 19).

We will experiment with the media composition, and particularly various ratios of nitrate ammonia, and urea. PH will be routinely measured in both open and closed culture environments. We will also experiment with buffer changes in the media, particularly various ratios of the bicarbonate and phosphate buffer systems.

Objective Four: Measurement of O_2, CO_2, and pH (20, 21):

We have budgeted the acquisition of a general measurement system (36) suitable for amperometric, potentiometric, and thermal sensing modules and electrodes (please see budget justification). This system, and the appropriate electrodes, will enable us to perform measurements of pO_2 and pCO_2, as well as nitrate, ammonia, and phosphate in various culture environments using the appropriate specific ion electrodes (32). Gas phase CO_2 will be measured by standard IR absorption methods.

The PI has worked extensively in the area of biosensors (see biosketch) and in the development and application of biochemical sensors, using optical analysis techniques. It may be possible that some of these newer prototype biosensors may also be employed in this project.

Objective Five: Area/Volume Effects:

Our present experience is based primarily on a small, 3-inch by 4-inch polyethylene bag containing about 30 ml of Guillard f/2 medium of pyrocystis lunula, with about 10 to 20 ml of enclosed gas volume. Such a container will maintain pyrocystis lunula cultures for up to 6 months when maintained under ambient lighting and room temperature conditions. Viability has been assessed by the qualitative measurement of bioluminescence intensity, and in a few cases by direct cell observation in the optical microscope.

Surface to volume ratio analysis will be performed analytically, using the techniques developed in a preliminary fashion by Foote (22). In addition to surface-to-volume ratio studies using a typical bag geometry, we will perform preliminary experiments with more unique bag geometries, including elongated bags, cylindrical and and spherical geometries.

Solution volumes will range from 10 to 5,000 ml, depending on the strength and mechanical characteristics of the bag materials and means of supporting the bags.

Objective Six: Other Phytoplankton:

Most of our experience to date has been with pyrocystis lunula. We also maintain pyrocystis noctiluca in the laboratory, and will study this organism as well. By discussions with dinoflagellate experts (15, 16), we will select a number of additional organisms, including the very large pyrocystis fusiformis, possibly gonyaulux polyedra, which is much more delicate in its culture requirements, and a number of others. The goal will be to see just how versatile and general, and how widely applicable, CO_2 permeable containers are for the culture of dinoflagellates and other microalgae.

We will also do some preliminary work on mixed cultures, initially with pyrocystis lunula and pyrocystis noctiluca. It may well be that mixed culture studies could even lead to the development of more complex ecosystems, possibly containing zoo-plankton, bioluminescent copopods, and other organisms

6. Related R and D:

This has already been discussed in sections 1 and 2 above. Protein Solutions, Inc. is committed to the science and application of bioluminescent dinoflagellates and other bioluminescent organisms for a variety of science education, biotechnological, and biomedical purposes. See also section H, Current and Pending Support, regarding PSI's other SBIR activities.

PSI has already invested about $60,000 in preliminary research on bioluminescent dinoflagellates for science education.

In these preliminary studies, we have demonstrated that pyrocystis lunula can be cultured and maintained in nonlaboratory environments, including homes, classrooms, and offices. We have found that no special lighting conditions or sources are required--normal room lighting and normal room temperatures are satisfactory. We have found that no special containers are required, only that they be transparent and clean. We have found that the cultures can be maintained for up to 6 months in totally sealed containers, with no oxygen or CO_2 exchange (23, 24). We have found that the organisms exhibit no measurable acute toxicity and appear to be completely safe (28). We have found that their light/dark cycle/circadian rhythm can be very easily altered and programmed. We have found that they can be shipped by first class mail across the country with little difficulty, as long as they are delivered and opened within 3 days.

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D. KEY PERSONNEL

1. Description:

J.D. Andrade, Ph.D., President and Chief Scientific Officer of Protein Solutions, has been working on bioluminescence for the past 5 years and on bioluminescent dinoflagellates for the past 2 years. He has supervised all of the work to date in the area of bioluminescent dinoflagellates. He has conducted courses for inservice elementary and high school teachers and has distributed pyrocystis lunula cultures to at least 50 different teachers, friends, co-workers, and advisors for their input and critique. Joe has a strong interest in integrated science education. Dr. Andrade has been working on biomaterials and biotechnology problems for the past 25 years. He is an accomplished scientist with 5 books and over 100 peer-reviewed publication. Joe is also Director of the University of Utah's Center for Integrated Science Education (CISE).

Although Joe is on the faculty of the University of Utah, he will be employed at Protein Solutions, Inc. for more than half time during the period of this SBIR (see letter on next page).

A part-time graduate student will perform much of the research work, under Andrade's direct supervision. Mr. John Tobler is an undergraduate biology student who has worked for PSI for the past two years. John is very experienced with dinoflagellate cultures and will be responsible for the more routine measurements and studies.

Advisors:

Dr. J.W. Hastings, Professor of Biology at Harvard University is one of the world's experts on dinoflagellate bioluminescence. Dr. Hastings serves on PSI's Scientific Advisory Board.

Another member of PSI's Scientific Advisory Board is Mr. William Kelley. Mr. Kelley was one of the founders of Aquarium Systems, a corporation that sells artificial seawater and related materials for hobbyists and exhibition aquaria. Mr. Kelley has also been involved in the design and development of exhibition aquaria nationally, including the Mystic Marine Life Aquarium in Mystic, Connecticut. He has retired in Salt Lake City and has been of immense help in the area of culture media for dinoflagellates.

Other dinoflagellate experts with whom Dr. Andrade has already consulted over the last 2 years and whom have offered their advice include Dr. Barbara Prezelin, Department of Biological Sciences, University of California, Santa Barbara; Dr. L. Brand, University of Miami, School of Oceanography, Miami; Dr. James Morin, Professor of Biology, , Los Angeles; Dr. Robert Guillard, the National Phytoplankton Culture Facility, West Boothbay Harbor, Maine. Refer also to Section F: Consultant