Idaho Incubation Fund Program

Bi-Annual Progress Report Form

Proposal No.	**IF13-002**
Name:	**Kenneth Cain**
Name of Institution:	**University of Idaho**
Project Title:	**Commercializing specific "naturally occurring" probiotic bacterial strains as feed additives to improve fish health and aid in disease management for aquaculture**

Information to be reported in your progress report is as follows:

1. Provide a summary of project goals/milestones for the period just completed, accomplishments for the period just completed, and plans and goals for the coming quarter:

Progress associated with specific proposal objectives are provided below:

Objective 1: Complete remaining objectives from FY12 funding

Ferment and lyophilize C6-6

Methods and Results:

C6-6 was grown in different media types to determine the best growth media for use in the fermentor. The media types tested were Luria-Bertani media (LB), 2 times tryptone yeast media (2xTY), terrific broth (TB), nutrient media (NM), and tryptic soy broth (TSB). Cultures of C6-6 were started from frozen stock in 15 mL of each of the media types and grown for 24 hrs at room temperature. Triplicate flasks of each media type containing 250 mL of media were then inoculated at a rate of 1% with the 24 hr culture from the corresponding media type. Cultures were shaken at 85 rpm at room temperature and optical density at 600nm was recorded every 30 min for each flask for 12 hours (Figure 1). Cultures grown in TSB and TB exhibited significantly higher growth rates than all other media types but and were not significantly different from one another. We determined TSB to be the best candidate for use in the fermentor as it provides equal growth and is less expensive than TB.

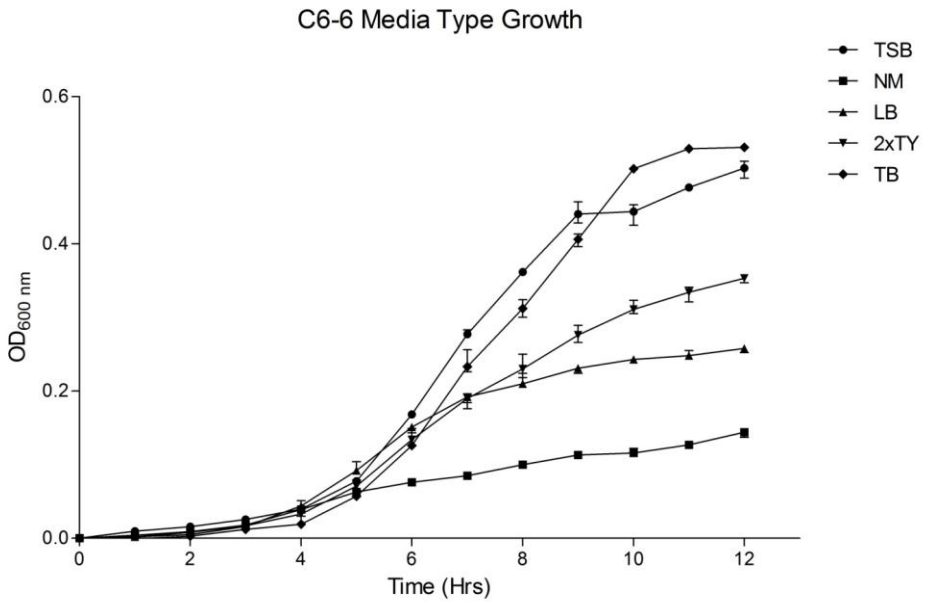

Figure 1 Growth of C6-6 in Luria-Bertani media (LB), 2 times tryptone yeast media (2xTY), terrific broth (TB), nutrient media (NM), and tryptic soy broth (TSB) at room temperature on 85 rpm shaker

Large scale production of C6-6 was tested in a 10 L fermentor. A 250 mL culture of C6-6 was started from frozen stock and incubated at 30°C for 24 hrs. The fermentor was filled with 8 L of TSB and inoculated at a rate of 1% with the 24 hr culture and 0.01% with Tween 20 as antifoam. The fermentor was maintained at 30°C with internal aeration and 200 rpm impeller agitation. Triplicate samples of the culture were collected every 30 min and the optical density at 600 nm was determined for 7 hours. C6-6 exhibited exceptional growth under these conditions and was determined to have an optimal harvest time of about 6 hrs post inoculation (Figure 2). While C6-8 was not tested in the fermentor, however, the similarities between the two species suggest that it would also perform well under these conditions.

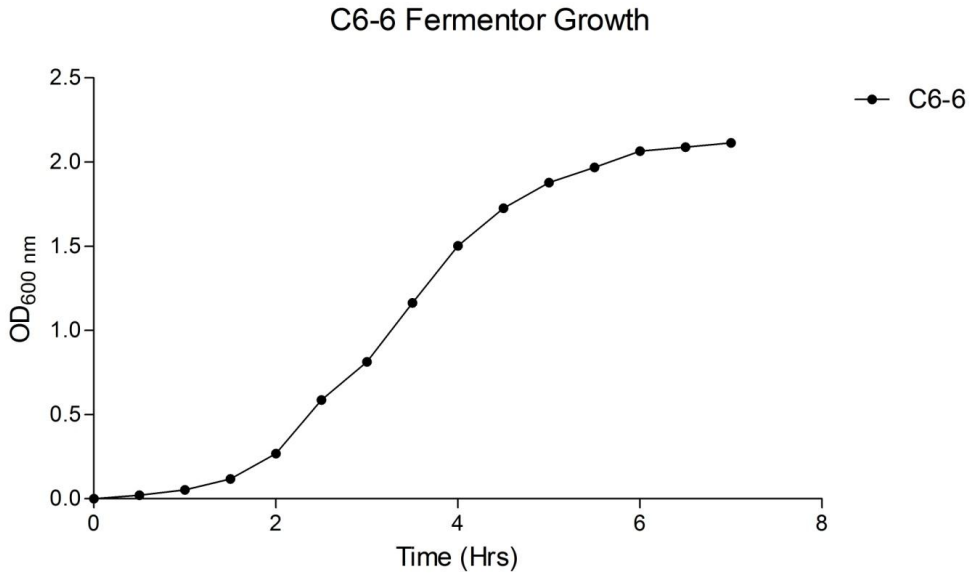

Figure 2 Growth of C6-6 at 30°C in fermentor

Methods to improve the survival of C6-6 during lyophilization were also investigated. Four different combinations of bacterial concentration and cryoprotectant were tested following production of C6-6 in the fermentor. Bacteria were centrifuged and resuspended in volumes of TSB and a cryoprotectant that resulted in a 10 or 20 times concentration of the original product. Bacteria were resuspended in TSB containing 10% non-fat dry milk or TSB containing 20% non-fat dry milk. The product was then lyophilized for three days at -60^{0}C. Survival was determined by comparing bacterial concentrations pre and post lyophilization using a drop plate method (Figure 3). Survival was greatest when the bacteria was concentrated 20 times and resuspended in TSB containing 20% non-fat dry milk.

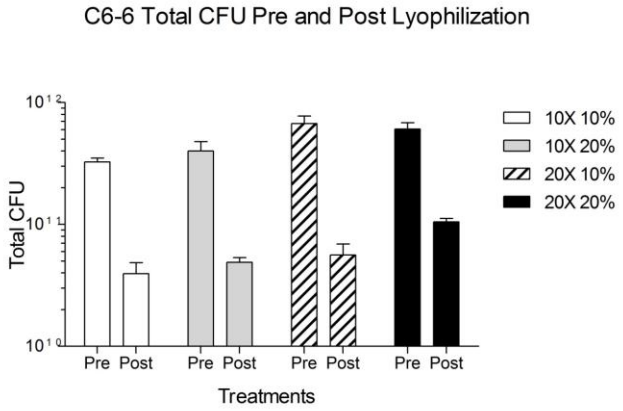

Figure 3 Average total colony forming units (CFU) of C6-6 pre and post lyophilization using various bacterial concentrations and amount of cryoprotectant

Summary:

We have shown that C6-6 can be grown and preserved with relative ease under laboratory conditions. Using common and relatively inexpensive growth media C6-6 can reach concentrations suitable for harvest within 5-6 hours of inoculation. The bacteria is also well suited to preservation requiring only food safe non-fat dry milk as a cryoprotectant making the preserved product ideal for addition fish feed.

Objective 2: Initiate laboratory and field trials to determine effectiveness of utilizing probiotics in a combined strategy (probiotics followed by vaccination)

- **Laboratory and field trials at trout hatcheries in Utah**

Methods and Results:

Rainbow trout were received at the UI from the Utah Division of Wildlife Resources as eyed eggs. At the time of hatching fish were assigned to one of four probiotic feed treatments, C6-6, C6-8, C6-6+C6-8 or oil (negative control). Probiotic treatments were administered beginning at first feeding. All fish remained on their respective diet until they were approximately 1 g in weight. At this time they were transitioned to a commercial diet. After 7 days of feeding the commercial diet, fish were vaccinated with commercially produced *F. psychrophilum* CSF 259-93 B.17 strain or mock vaccinated with sterile triptone yeast extract salts (TYES) culture media replacing the vaccine. Fish were dip vaccinated for 90 seconds in a 1:10 dilution of the commercial vaccine or sterile TYES in tank water. This method mimics the method used in vaccine field trials. Two weeks after initial vaccination all groups received a booster vaccination or an additional mock vaccination using the same methods. All fish were then challenged with virulent *F. psychrophilum* CSF 259-93 four weeks after the booster vaccination and mortality was monitored for 28 days. Blood was collected prior to booster vaccination and challenge and analyzed to determine the production of *F. psychrophilum* antibodies.

Cumulative percent mortality (CPM) for all groups is presented in Table 1. Overall CPM was high and ranged from 64.9 ± 7.0 in the C6-8 + mock vaccination treatment to 89.8 ± 2.0 in the Oil + mock vaccination treatments. However, there were no significant differences in the CPM between any of the groups.

Table 1 Cumulative percent mortality (mean ± SEM) following challenge with *Flavobacterium psychrophilum* of rainbow trout fry after feeding of probiotics and vaccination with live *F. psychrophilum* CSF 259-93 B.17

Treatment	CPM
Oil + mock vaccination	89.8 ± 2.0
C6-6 + mock vaccination	74.0 ± 9.5
C6-8 + mock vaccination	73.7 ± 3.0
Combined + mock vaccination	85.5 ± 1.2
Oil + vaccination	83.0 ± 7.0
C6-6 + vaccination	68.6 ± 7.2
C6-8 + vaccination	85.0 ± 2.1
Combined + vaccination	76.0 ± 1.2

Mortality trends were analyzed using a log rank survival curves. Significant differences ($P < 0.05$) in survival curves were observed between the oil + mock vaccination and the C6-6 + vaccination treatments. No other groups were significantly different from one another (Figure 3).

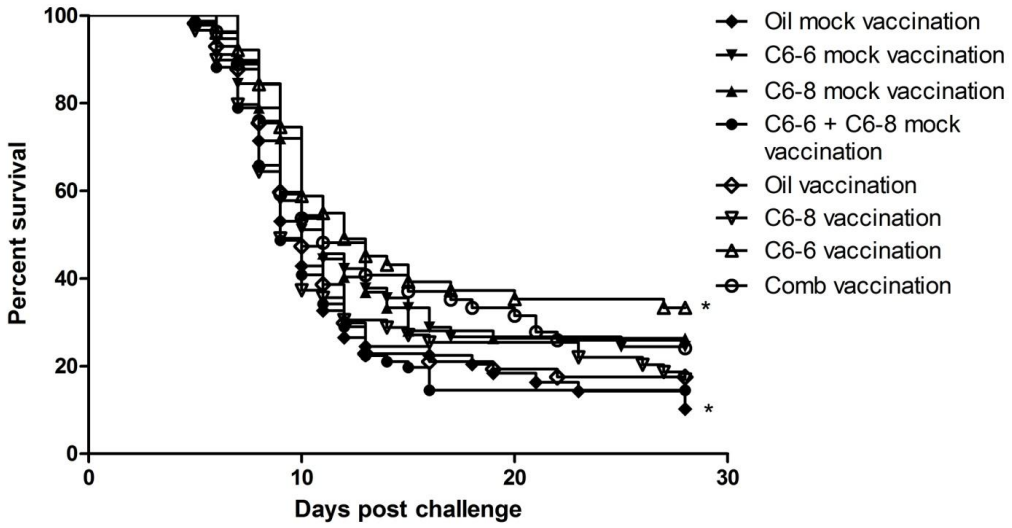

Figure 3 Survival curves of rainbow trout fry following challenge with *Flavobacterium psychrophilum* after feeding of probiotics and vaccination with live *F. psychrophilum* CSF 259-93 B.17. Significant differences ($P < 0.05$) between treatments in log rank survival curves are noted with (*).

Antibody titers remained below the detectable limit for all groups following initial vaccination and booster immunization.

Parallel filed trials were conducted using the same stock of fish at Utah's Fountain Green hatchery. The trial was monitored by Utah Division of Wildlife personnel.

Summary:

Laboratory trial:

In the laboratory trial, mortality following *in vivo* challenge was higher than anticipated. This was likely due to the requirement of direct subcutaneous injection of *F. psychrophilum* into fish, which often can be problematic and lead to more severe disease outbreaks than desired. Unlike a natural infection, where exposure is achieved through contact with water or other fish containing the bacteria, laboratory challenges bypass important immune barriers such as the skin and mucosal surfaces. As it is currently unknown how the vaccine and probiotics provide protection against *F. psychrophilum*, it is possible that this injection challenge was too severe. This makes interpretation of results and their relationship to a hatchery situation difficult.

The antibody titers following vaccination remained below our detection limits. It is likely that the lack of antibody response corresponds to the vaccine delivery method used in this study. A 90 second immersion in a 1:10 dilution of the vaccine may not be sufficient to stimulate a strong immune response due to low bacterial concentrations and a short exposure time. Based on this assumption the effectiveness of the vaccine may be increased by increasing the bacterial concentrations or exposure time.

Despite the high levels of mortality and lack of antibody response following vaccination we did observe significantly different survival curves between the C6-6 + vaccination and the oil + mock vaccination treatments. This indicates that the combination of the probiotic and vaccination was able to slow the rate of mortality. As this difference was not observed in any of the other vaccinated groups or the group that was administer C6-6 + mock vaccination it is likely that the combination of the two treatments has the potential to be more effective that either treatment alone.

With the ease in which C6-6 can be grown and preserved and its increased benefits when combined with vaccination it has shown a strong potential to further decrease hatchery mortality caused by *F. psychrophilum* infections.

Field trial:

The parallel field trial in Utah had mixed results. With the exception of one tank of fish, there was no CWD outbreak during the 30 day feeding of probiotics prior to vaccination. However, CWD was confirmed in fish approximately 1.5 months following vaccination. No distinct trends could be observed except that all fish receiving the vaccine had delayed mortality due to CWD. Two groups of vaccinates (with pre treatments of

probiotics) showed distinct reduction in mortality over corresponding controls. Nevertheless, antibiotic treatments had to be applied due to the need to meet production targets.

Taken together the data generated from the lab and field trial suggests that a 30 day feeding of probiotics prior to vaccination may not provide the anticipated benefits we expected. However, this should be interpreted cautiously as we do not fully understand the mechanism associated with protection conferred by the probiotics, and we have not fully analyzed the data. Current trials are addressing the potential role of the immune system and how it may or may not be stimulated following probiotic delivery.

Objective 3: Determine if C6-6 and/or C6-8 are effective at reducing mortality due to columnaris in warmwater aquaculture species.

Materials and Results:

The C6-8 strain was selected for use in *in vivo* studies because both the cells and the supernatant from this probiotic inhibited or reduced the growth of a virulent channel catfish isolate of *Flavobacterium columnare*. Based on these *in vitro* results, a feeding trial was performed to determine if delivery of the probiotic C6-8 via feed reduces mortality following *F. columnare* challenge in channel catfish. In the trial, two groups of channel catfish (~ 3.0 g) were fed for 10 days with either the treatment, consisting of standard feed top dressed with menhaden oil containing the probiotic, or the control, consisting of standard feed top dressed with menhaden oil (without probiotic). After the 10 day feeding period, triplicate groups of 25 fish from treatment and control groups were challenged with a high and low dose of *F. columnare*, and mortalities were monitored for 11 days. At the high challenge dose, the mean mortality ± SEM of the control group was 68 ± 15 %, while the mortality of the treatment group was 55 ± 24%. At the low challenge dose, the mean mortality ± SEM of the control group was 23 ± 11 %, while the mortality of the treatment group was 11 ± 4%.

Summary:

These results indicate a potential beneficial effect (i.e., reduced mortality) of feeding the C6-8 probiotic strain prior to *F. columnare* challenge, but there were no significant differences between the treatment and control groups. Based on these *in vivo* results, an additional feed trial will be performed to determine if feeding the probiotic C6-8 for a longer duration prior to *F. columnare* challenge enhances this beneficial effect.

2. Provide a summary of budget expenditures for the period just completed:

Budget expenditures were primarily for salary, travel, and supplies.

3. List patents, copyrights, plant variety protection certificates received or pending:

- Probiotic bacterial strains and method of use to decrease mortality in fish due to bacterial disease. PCT/US12/29896 - filed on March 21st, 2012 by the UI and published on October 11, 2012.

4. List invention disclosures, patent, copyright and PVP applications filed, technology licenses/options signed, start-up businesses created, and industry involvement:

    - Discovery of specific probiotics bacterial strains capable of reducing disease related mortality in aquaculture – Invention Disclosure filed 1/4/2011

    - Met with the company Aquatic Life Sciences on 12/12/12 to discuss commercialization potential and strategize for regulatory approval of product.

5. Include funding burn rate:

    - Since funding became available after July 1st, 2012, a total of approximately $41,000 has been spent.

6. Any other pertinent information: