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

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.

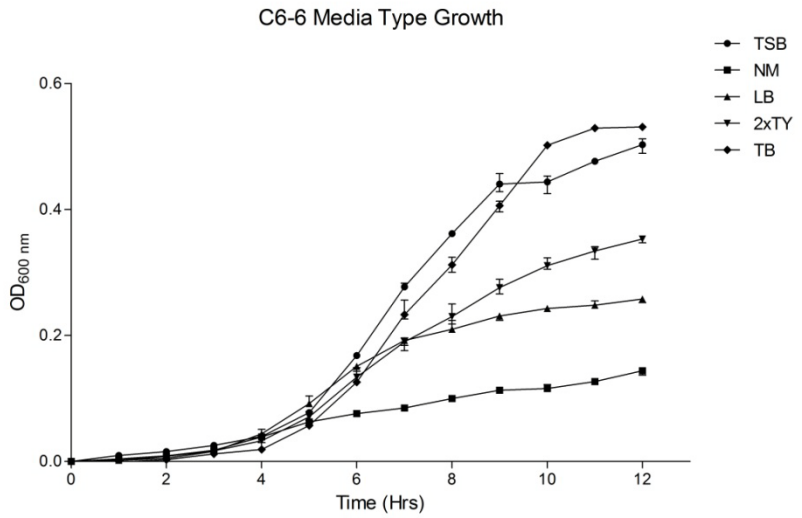

Figure 1 Growth of C6-6 in Luria-Bertani media (LB), 2 times tryptone yeast media (2xTY), terrific broth (TB), nutrient media (NM), and tryptic soy broth (TSB)

Large scale production of C6-6 was evaluated 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.

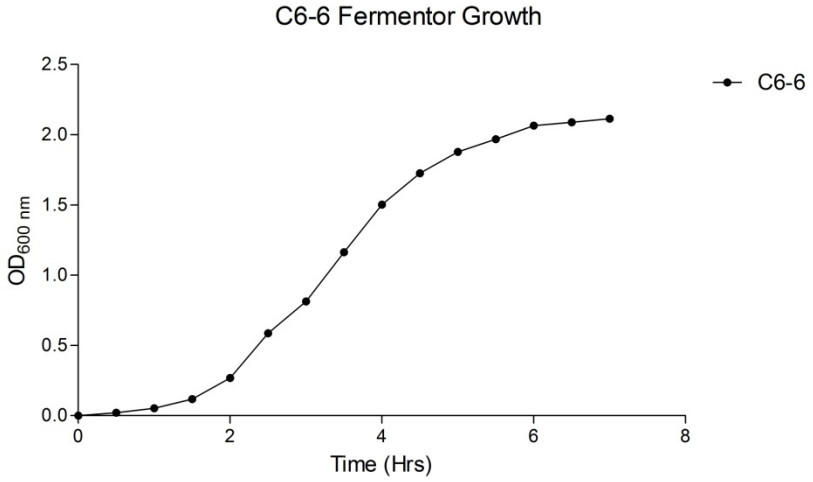

Figure 2 Growth of C6-6 at 30°C in fermentor

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 resuspened in volumes of TSB and a cryoprotectant that resulted in a 10 or 20 times concentration of the original product. Bacteria were resuspened 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 -60ºC. 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 resuspened in TSB containing 20% non-fat dry milk.

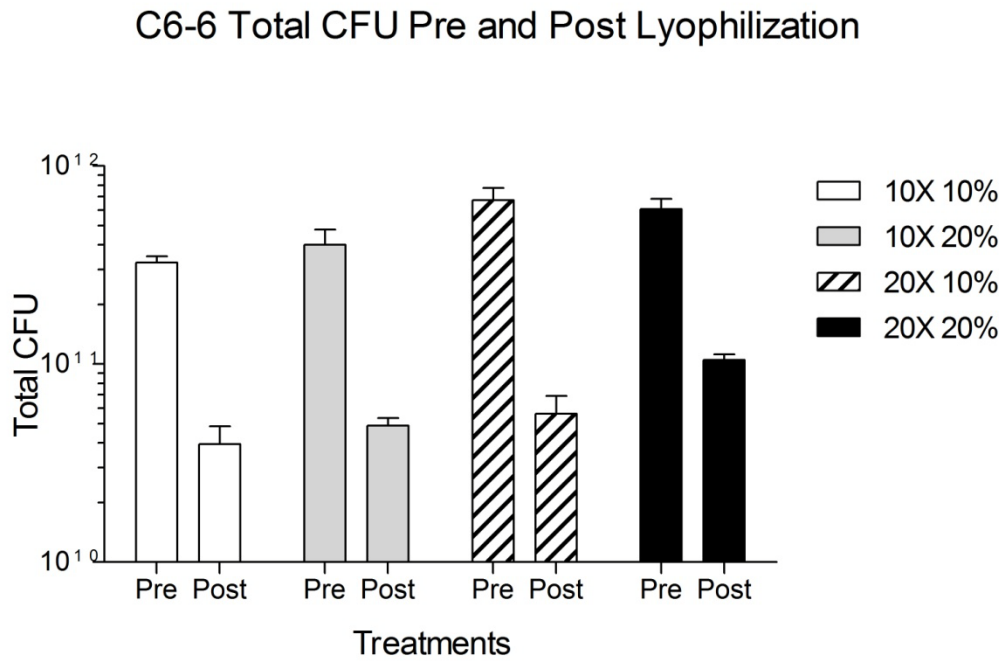

Figure 3 Average total colony forming units (CFU) of C6-6 pre and post lyophilization

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 (see letter of support)**

To evaluate the effects of combined administration of C6-6 and C6-8, rainbow trout have been obtained from the Utah Division of Wildlife Resources as eyed eggs for laboratory experiments. At first feeding fish were administered one of four treatment diets coated with fish oil containing C6-6, C6-8, C6-6+C6-8 (combined) or oil alone(negative control). These fish were challenged with *Flavobacterium psychrophilum* on December 17th and will be monitored for mortality for 28 days.

To evaluate the effects of combining probiotic treatment with vaccination rainbow trout were received from the Utah Division of Wildlife Resources as eyed eggs. Fish were then assigned one of four probiotic treatments, C6-6, C6-8 C6-6+C6-8 (combined) or oil (negative control). Probiotic treatments were administered beginning at first feeding. On December 18th half of each treatment will be vaccinated via immersion in the attenuated *F. psychrophilum* stain CSF 259-93 B.17 as the vaccinate group. The other half received a mock vaccination by immersion in sterile culture media and will serve as the negative control. The groups will then receive a booster vaccination or an additional mock vaccination two weeks after primary vaccination using the same methods. Fish will then be injection challenged with *F. psychrophilum* or mock infected with PBS four weeks after booster vaccination and mortality will be monitored for 28 days. Blood will be collected at two week intervals following initial vaccination and used to determine if there are any differences in antibody production between the probiotic treatments.

Parallel field trials at Utah State hatcheries were initiated in December and those groups are scheduled for vaccination in mid January.

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

The *in vitro* inhibition assay used for *Flavobacterium psychrophilum* has been optimized for use with *Flavobacterium columnare*, which requires different bacteriological growth medium and culture temperature. Initial work has demonstrated that cells from the C6-6 strain inhibit or reduce the growth of a virulent channel catfish isolate. The same studies have been conducted using the C6-8 strain and the research has demonstrated that both the cells and the supernatant from this probiotic inhibit or reduce the growth of the channel catfish isolate. Research is underway to determine whether these probiotic strains inhibit the growth of an isolate of *F. columnare* that is virulent for tilapia. Based on these *in vitro* results, preliminary feed trials have been initiated and are ongoing.

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 April 5th, 2012 by the UI.

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 $14, 584 has been spent.

6. Any other pertinent information: