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The Role of Natural Products in Acidovorax delafieldii ATH2-2RS/1 Survival in a Dry Formulation

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Honors Thesis

The Role of Natural Products in *Acidovorax delafieldii* ATH2-2RS/1 Survival in a Dry Formulation

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Abstract

If rhizobacteria are to be used for weed biocontrol, a practical means of storing them must be found. In this project I sought to use the natural products exopolysaccharide and hydroxyectoine to improve the survival of *Acidovorax delafieldii* dry formulated with either an oat flour, maltose, and trehalose formulation or Celite (diatomaceous earth). When formulated with the oat flour, maltose, and trehalose, the expolysaccharide allowed for modest survival after 24 hours of drying. Using Celite, a preferred carrier, hydroxyectoine led to bacterial populations of $6.2x10^4$ cells/g formulation after 24 hours of drying and 48 hours of cold storage.

Introduction

It is becoming increasingly common to utilize more natural, organic methods of suppressing the growth of weeds or fostering the growth of crops instead of using traditional, often harmful, herbicides, pesticides, and fertilizers. The field of biocontrol, which uses insects and micro-organisms to control weeds and pests, is growing rapidly, and a variety of organisms have been found that can target specific weeds. However, biocontrol agents are of little commercial use without an economically feasible means of storage, delivery, and application. Like all living organisms, water is essential to the survival and proliferation of bacteria. Desiccation is the primary issue that makes it difficult for bacteria to survive in a dry formulation, and for decades it has been known that natural products, or molecules produced by living organisms, can promote desiccation survival (Kloepper 1981). More recent studies have demonstrated that exopolysaccharides can help prevent desiccation in other species of bacteria (Klock et al. 2007, Slininger et al. 2010). Exopolysaccharides (EPS), large polymers of sugar molecules secreted by many types of bacteria and even some fungi, have been found to help bacteria survive drying by promoting water retention (Roberson & Firestone 1992, Sayyed et al. 2011). Hydroxyectoine, a substituted pyrimidine, has also been effective in increasing the dry storage survival of *Pseudomonas Putida* (Manzanera et al. 2002).

Since there is good evidence that both exopolysaccharide and hydroxyectoine benefit the dry formulation survival of other bacteria, I endeavored to determine whether this same benefit can be imparted to a specific species of rhizobacteria. In my project, I sought to improve the survival of *Acidovorax delafieldii* ATH 2-2RS/1 (RS) bacteria in dry formulation through the addition of one of two different natural products: either exopolysaccharide or hydroxyectoine.

Methods:

Exopolysaccharide Production and Isolation

The first step of this project was to produce and isolate the exopolysaccharide from *Rhizobium rubi.* It has been found that Yeast Extract Mannitol (YEM) broth, made from 0.4 g yeast extract, 10 g mannitol, 0.5 g dipotassium phosphate, 0.2 g magnesium sulphate heptahydrate, and 0.1 g sodium chloride per liter, autoclaved and pH balanced to 6.8, optimizes EPS production in *Rhizobium* species organisms (Sayyed et al. 2011). One liter YEM broth was inoculated with *R. rubi* and placed in a 28°C shaking water bath for three days. The culture was then transferred to sterile bottles and centrifuged for 15 minutes at 7000 rpm, and the EPS-containing supernatant was poured off and filtered. The obtained solution was then precipitated with two volumes of 2-propanol, chilled, and then centrifuged again for 15 minutes at 7000 rpm to concentrate the EPS. The supernatant was poured off and the isolated EPS was suspended in minimal distilled water. This process of precipitating with two volumes of 2-propanol was repeated twice more to further purify the EPS. The resulting EPS suspension was then put into dialysis tubing and dialyzed against distilled water overnight. This final, purified EPS solution was stored frozen until needed.

Exopolysaccharide and Dry Formulation Survival

To study the influence of EPS on RS dry formulation survival, 100 mL tryptic soy broth (TSB) was prepared using 30 g tryptic soy powder per liter of water, autoclaved 22 minutes, inoculated with RS, and put in a 28°C water bath for two days. The culture was then transferred to centrifuge tubes and spun on high for 20 minutes to pellet the cells. The supernatant was removed and the cells were resuspended in one tenth the initial volume using TSB.

To prepare the dry formulation, 1 g maltose, 4 g oat flour, and 0.1027 g trehalose were weighed out and mixed in a flamed mortar and pestle. To this dry formulation 0.5 mL RS

suspension and either 0.5 mL of the EPS or 0.5 mL of sterile water was added. The liquid was thoroughly mixed with the dry substrate then transferred to a petri plate and dried in a 28°C incubator for 24 hours.

After drying, the bacterial survival was ascertained by performing a spread plate dilution series; 0.1 g of the dry formulation was suspended in 10 mL of 0.1% peptone broth and mixed for two minutes. Dilutions were performed by pipetting 1 mL from each tube into a test tube with 9 mL peptone broth, and 0.1 mL aliquots of these solutions were plated in triplicate on ½ tryptic soy agar plates. Half of the plates contained 100 μg/mL rifampicin, an antibiotic for which this strain of RS is resistant, while half of the plates lacked the antibiotic.

Hydroxyectoine and Dry Formulation Survival

In this experiment, the RS was cultured and concentrated as in the previous section. 0.5 g Celite was put in a petri plate, and 0.1 mL bacterial suspension, either with or without 0.0158 g hydroxyectoine (to equal 1M final concentration), was mixed thoroughly with it. This formulation was then dried in a 28°C incubator for 24 hours, then transferred to a 4°C refrigerator for an additional two days. At the end of this timeframe, the entire dry formulation was resuspended in 20 mL 0.1% peptone broth, and shaken for 10 minutes at 140 rpm. 0.1mL portions of this suspension and dilutions were plated in triplicate.

Data Recording and Analysis

Growth on the plates was determined after 3-4 days, when colonies were large enough to be counted. Colony appearance and Gram stains helped to confirm that the colonies resulting on the plates were surviving RS and not contamination. Each experiment was performed twice, in duplicate, for a total of four identical trials. To determine whether there was a statistically significant

difference between the resulting bacterial populations from the dry formulas with or without the natural product added, an unpaired two-tailed T-test was performed for each trial and the resulting probability values are reported.

Results

In the experiments where exopolysaccharide and oat flour, maltose, and trehalose were used, low repeatability was obtained. There was high variation in bacterial populations between and within experiments (fig. 1). The result in the first replicate of experiment 1 was not statistically significant, while the second replicate, and both of the replicates in experiment two were statistically significant ($p<.05$). On average for both experiments, addition of EPS significantly raised the survival rate to 3100 colony forming units per gram (cfu/g) from 2300 cfu/g without EPS.

The results of the trial with hydroxyectoine and Celite trial are more conclusive. In the trials without hydroxyectoine, bacterial survival was below the threshold of detection (<400 cfu/g Celite). When hydroxyectoine was added, mean populations of 1.3×10^4 for the first experiment and 1.1×10^5 cfu/g for the second experiment were obtained, for an average result of 6.2×10^4 cfu/g (fig. 2). While the replicates within each group were consistent with one another, there was nearly a tenfold difference between the survival of the first and second experiments. T-tests on the replicates in both experiments confirmed that these results are significantly greater than the absence of detectable survival in the group without hydroxyectoine.

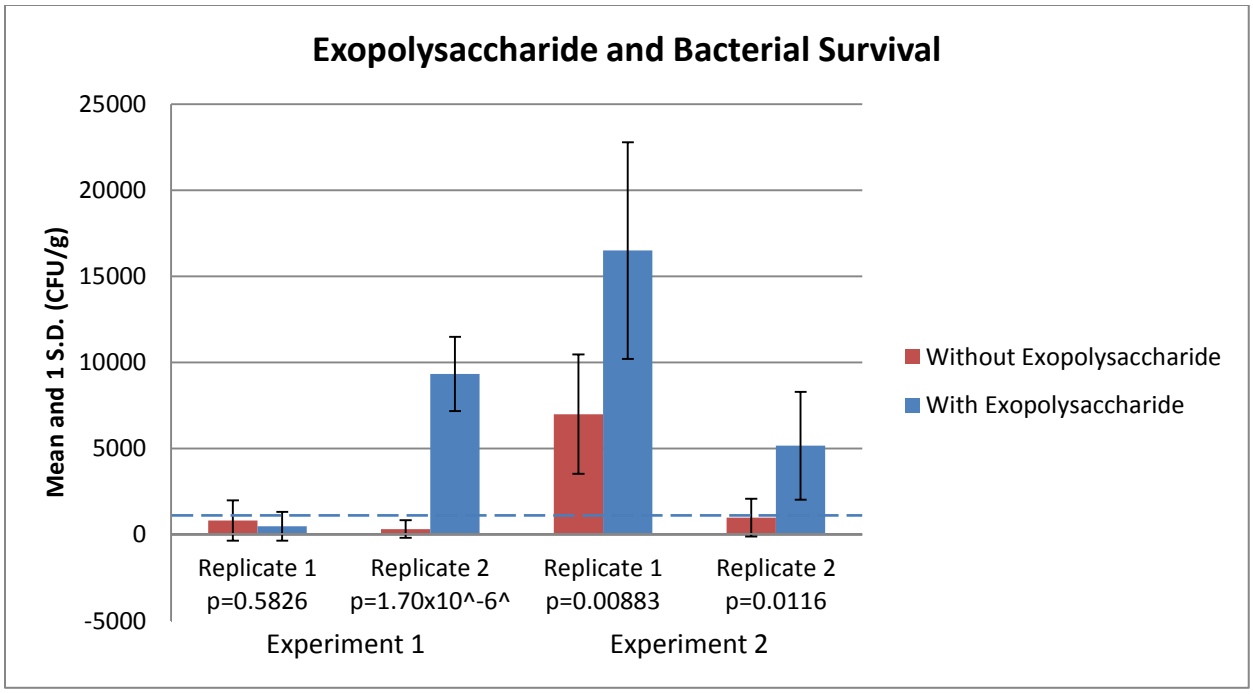

Figure 1. *A. delafieldii* ATH2-2RS/1 populations recovered from an oat flour, maltose, and trehalose dry formulation with and without exopolysaccharide after 24 hours at 28°C. Each bar represents the average colony forming units per gram (cfu/g) in the surviving bacterial population on six plates, while the error bars indicate ±1 standard deviation. The threshold of detection, 1000 cfu/g, is indicated by the dashed line on the graph. The p values of an unpaired t-test are presented with each replicate.

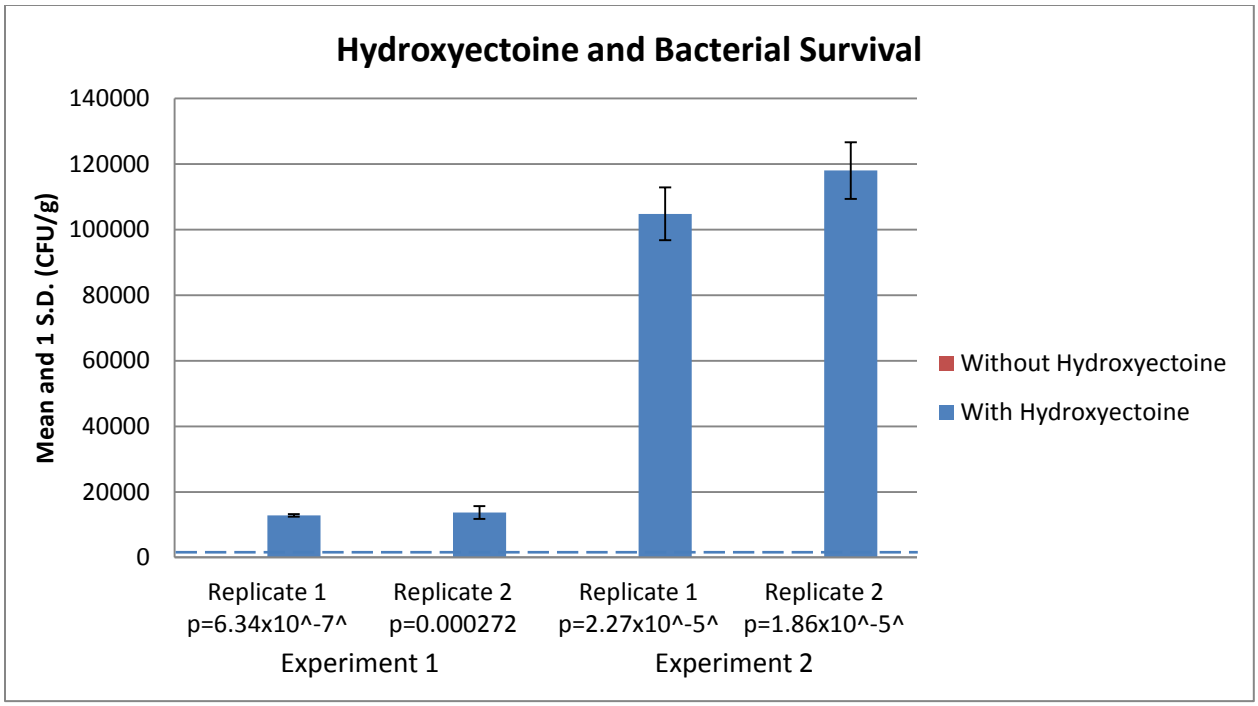

Figure 2. Populations of *A. delafieldii* ATH2-2RS/1 recovered from a dry formulation of Celite with and without hydroxyectoine after 24 hours at 28°C and 48 hours at 4°C. Each bar on the graph represents the average population, in colony forming units per gram (cfu/g) of dry formulation, as recorded from three plates, and the error bars indicate ±1 standard deviation. The dashed line on the graph denotes the threshold of detection, 400 cfu/g. An unpaired t-test was conducted on each replicate, and the p values are presented on the graph. None of the formulations without hydroxyectoine yielded detectable populations.

Discussion

Overall, the results of this project answered the question I was initially asking; both exopolysaccharide and hydroxyectoine can help RS survive in a dry formulation. As observed in figures 1 and 2, while EPS significantly increased RS survival in three of four trials, hydroxyectoine had a more significant effect. The effect of EPS on RS dry formulation survival appears to be minor, as when I tried formulating the RS with EPS and Celite, no survival was obtained, so hydroxyectoine seems to be the superior natural product. Something else worth noting when comparing the results of the two natural products in this project is that in the experiment with hydroxyectoine, the bacteria were subjected to the additional stress of two days storage at 4°C that the EPS trial didn't involve, and even with this the hydroxyectoine enabled higher survival.

Some of the variation in the experiments with EPS was likely due to sampling errors. 5 g of dry formulation was prepared, and only 100 mg of the dry formulation was resuspended and checked for surviving bacteria. If the RS added to the dry formulation were not mixed perfectly well with the oat flour, maltose, and trehalose dry formulation, the recovered populations could vary quite significantly from both the actual survival and the results of other replicates of the same experiment. Even so, some of the variation may simply be the nature of the minor effect of the EPS. The differences in survival between the two hydroxyectoine experiments suggest that *A. delafieldi* is incredibly sensitive to desiccation. There was a very small deviation in the precise amount of time the formulation dried at 28°C; in the first experiment, it was a little over 24 hours, while in the second, it was a little under. This accounts for the differences between the two experiments.

In Bonnie Cho's honors project (2004) in which she investigated an ideal pesta dry formulation for RS, she found that the oat flour, maltose, and trehalose formula I used in the EPS study yielded a maximum RS survival of 1.1×10^4 cfu/g. This is slightly higher than I obtained, even with EPS added. However, when formulated with Celite (which is harder on RS survival as it has no

nutrient component) in the presence of hydroxyectoine, higher survival was obtained. This again demonstrates the effect of the hydroxyectoine. When formulating *P. putida* with hydroxyectoine, Manzanera et al. (2002) obtained survival of 40-60% for up to 42 days. This is very significant survival and demonstrates that hydroxyectoine works well in some cases.

Roberson and Firestone (1992) found that EPS greatly decreases the rate at which water is lost from around the bacteria, and thus the presence of EPS helps prevent desiccation. In their research with *Pseudomonas fluorescens* and EPS, Slininger, Dunlap and Schisler (2010) found that in some formulations, EPS significantly favoured bacterial survival, enabling levels of 10^6 and 10^7 cells/g Hyflo after four months storage. While the numbers I've obtained seem low in comparison, much of the reason is because *A. delafieldii* is simply a less hardy bacterium and doesn't handle the stress of desiccation as well.

There is certainly opportunity for further research into these questions. One question is whether 1M is the optimum concentration of hydroxyectoine for promoting RS survival. Manzanera et al. (2002) noted that this was the optimum concentration in *P. putida*, but whether this is the ideal concentration in *A. delafieldii* is not known yet. Longer term trials to determine the effect of hydroxyectoine on RS survival over weeks of storage are needed. Perhaps there are other natural products that can also be tested that would give even better dry formulation survival results than hydroxyectoine and exopolysaccharide.

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