Spring 5-2-2020

Glycogen Accumulation by Wild Type and Bald Mutant Strains of Streptomyces sp. SFB5A during Growth on 3-Hydroxybutyrate

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Abstract:

Previous research has shown that *Streptomyces* sp. SFB5A produces PHA depolymerase and forms aerial hyphae, while the *bld4* mutant of this species forms aerial hyphae very poorly and does not produce PHA depolymerase. This effect may be due to the mutant's inability to sense starvation. Therefore, we hypothesized that the *bld4* may also be deficient in formation of glycogen and spores, which are both associated with starvation. To test this hypothesis, we grew the wild type (WT) *Streptomyces* sp. SFB5A and *bld4* in broth cultures containing 3HB. We compared 3HB consumption, glycogen accumulation, PHA depolymerase synthesis, protein accumulation, and spore accumulation in both strains over a seven-day period.

Introduction:

Members of the *Streptomyces* genus consist of filamentous, gram-positive bacteria that are responsible for the degradation of many organic polymers in the soil [1]. Members of this genus are also able to produce a range of secondary metabolites, including nearly half of all known antibiotics. For example, one metabolite of *Streptomyces coelicolor*, an aromatic polyketide actinorhodin (Act), is secreted in agar media as a blue pigment in neutral and alkaline environments but appears red in acidic environments [4].

The life cycle of *Streptomyces* is similar to that of fungi and consists of discrete stages of cellular differentiation. The cycle begins through the formation of a vegetative mycelium from a spore. Rather than producing separate cells during cell division, *Streptomyces* species form multinucleate cell chains of branched mycelium [4]. During the aerial mycelium phase, the hyphae begin to grow away from the base of the vegetative hyphae, which results in a fuzzy white appearance at the surface of the colonies [4]. The production of aerial mycelium in *Streptomyces coelicolor* and other members of the *Streptomyces* genus is controlled by the *bld* genes. Particular mutants of *Streptomyces coelicolor* that exhibit defects in morphological differentiation are called bald (*bld)* mutants [5]. The *bld* mutants are unable to form aerial hyphae in a rich medium and are defective in antibiotic production. The primary defect observed in *bld* mutants involves the regulation of carbon utilization. This suggests that the inability of *bld* mutants to commence morphogenesis may be caused by their inability to sense or signal starvation. [5].

Polyhydroxyalkanoates (PHAs) are polymers of 3-hydoxyalkanoate monomers and have potential use as biodegradable plastics [9]. The most common PHA is polyhydroxybutyrate (PHB), which is comprised of 3-hydroxybutyrate (3HB) monomers [9]. The organism used in Dr. Baron's lab, *Streptomyces* sp. SFB5A, produces a PHA depolymerase, which breaks down PHB and other PHAs into their monomers, which are in turn used for growth [1]. Prior research has shown that PHA depolymerase in strain SFB5A is synthesized during growth on PHB or 3HB but not glucose, which suggests transcriptional regulation of the PHA depolymerase gene (*phaZ*) [1].

The *phaZ* gene of *Streptomyces* sp. SFB5A has been cloned and sequenced, including its promoter region [1]. The promoter region was found to have DNA homology to a binding site for a transcriptional activator, SpoIIID from *Bacillus subtilis*. This protein activates genes for sporulation in response to carbon starvation [8]. By analogy, *Streptomyces* sp. SFB5A may transcribe the *phaZ* gene in response to carbon starvation and/or cellular differentiation, which might be activated by the binding of a SpoIIID analog to the promoter region.

Nutrient deprivation in *Streptomyces* is accompanied by the synthesis of glycogen, which is oftentimes accumulated in bacteria as an energy reserve during starvation conditions [2]. Glycogen deposits have been found in two locations in colonies: Phase I and Phase II [2]. Phase I glycogen deposits, which appear at the interface between vegetative and aerial mycelium, are thought to be used as a carbon and energy source to fuel the development of aerial mycelium [2]. Phase II glycogen is produced during the formation of pre-spores near the end of aerial mycelium development [6] and may provide much of the carbon for abundant components of mature spores, such as spore walls, spore pigments, and trehalose [2,6]. Phase II glycogen stores thus diminish as mature spores are formed [6]

The synthesis of PHA depolymerase in wild type (WT) *Streptomyces* sp. SFB5A during growth on PHB was recently shown to correlate directly with glycogen production, both peaking at 36 hours during formation of pre-spore compartments [3]. Based on the timing of PHA depolymerase synthesis and appearance of pre-spores, this is presumably Phase II glycogen. Moreover, during the spring of 2018, a bald mutant of *Streptomyces* sp. SFB5A, *bld4*, which also lacks the ability to produce PHA depolymerase, was studied [7]. This mutant grows well on glucose or 3HB, but poorly on PHB. When the WT and *bld4* were grown in glucose medium, glycogen production in *bld4* was compromised as compared to the WT, which produced significantly higher levels of glycogen from 24-72 hours [7]. In addition, the WT showed formation of short, rectangular pre-spores at 36 hours, while the *bld4* cells were longer and more filamentous, with some curling typical of aerial hyphae, and few pre-spores were observed. However, other indicators of normal cellular physiology, such as glucose metabolism rates and production of cellular protein were similar in both strains [7]. Since glucose does not support PHA depolymerase synthesis in *Streptomyces* sp. SFB5A, a direct link between nutrient deprivation (as indicated by glycogen accumulation) and triggering of PHA depolymerase synthesis could not be established.

Therefore, in a subsequent study, the WT and *bld4* were grown with 3HB, which supports good growth of both strains [7]. The WT, but not *bld4,* produces PHA depolymerase during growth in this medium [7]. Similar to the results with glucose, *bld4* was compromised in glycogen production during growth on 3HB, which indirectly supports the hypothesis that nutrient limitation might be the initial trigger for PHA depolymerase synthesis [7]. These studies were done by a medium replacement technique, whereby cells are grown in a nutrient broth, centrifuged, and resuspended in an equal volume of mineral salts medium. Therefore, the cells are already in a stationary phase, and the entire volume of the original growth medium is simply replaced with an equal volume of mineral salts medium, resulting in a concentrated cell suspension from the start of the experiment.

In the current study, the previous methods were compared to a lower inoculum method. The WT and the *bld4* mutant of *Streptomyces* sp. SFB5A were grown in a medium containing 3HB, but not using the medium replacement technique. Instead, the study began with a much lower percent inoculum of stationary phase cells (10% v/v), so that the cells must go through a distinct log phase of growth. As in previous research, glycogen production, 3HB consumption, PHA depolymerase production, cellular protein were monitored, to determine the effect of changing the inoculum size.

Methods:

Media and Culture Conditions: During this study, the WT of *Streptomyces* sp. SFB5A was grown overnight in 2mL of nutrient broth, and 600 µL of the sample was transferred into 30 mL of nutrient broth and was grown for 4 hrs, as determined by a growth curve analysis. The *bld4* mutant of *Streptomyces* sp. SFB5A was grown for 24 hrs in 2 mL of nutrient broth, and 600 µL of the sample was transferred into 30mL of nutrient broth and was grown for 5.5 hrs, as determined by a growth curve analysis to represent mid-logarithmic phase. Cells were harvested by centrifugation and resuspended in an equal volume of mineral salts liquid medium, Schegel's nitrogen complete (SNC) [7] containing 20 mM 3HB (denoted SNC-3HB). Flasks of SNC-3HB were inoculated with the resuspended cells at 10% v/v. The cultures were incubated with shaking at 30°C for seven days. Two samples, one at 5.6 mL and one at 1.4 mL each, were removed at 0, 24, 36, 48, 72, 96, 120, 144, and 168 hrs and were centrifuged. The supernatant and the pellet for each day were stored at -80ºC for later use.

Glycogen Assay: Intracellular glycogen levels were determined as glucose by a modification of the method of Chun and Yin [11]. Briefly, pellets were resuspended with 1 mL of deionized water, spun down for 2.5 min, and the supernatant was removed. 1.7 mL of 30% KOH was added and incubated at 100ºC for 20 min. 1.275 mL of ice-cold ethanol was added and centrifuged at 4,000 *x g* for 15 min. Supernatants were discarded. The glycogen pellet was resuspended in 500 µL of deionized water and 1 mL of 0.2% anthrone reagent was added (0.2 grams of anthrone in 100 mL of 98% H_2SO_4). Tubes were incubated at 100ºC for 10 min. Optical density was determined by a spectrophotometer at 620 nm and a glucose standard curve was performed.

Protein Assay: Protein concentration levels were determined by the Sigma bicinchoninic acid (BCA) protein assay. The optical density of a clean microtiter dish was determined at 562 nm as a control. After step three of the glycogen assay, spinning down the debris and adding 30% KOH, 25 µL was removed and placed in a sterile microtiter dish. 25 µL of deionized water was added to each sample. A well containing 25 µL of 30% KOH and 25 µL of deionized water was prepared as a reagent control. 200 µL of BCA working reagent was added to each well. The microtiter dish was incubated at 60 ºC for 15 min. Optical density was determined at 562 nm and a bovine serum albumin standard curve was performed.

PHB Depolymerase Assay: PHB depolymerase activity was determined by monitoring the decrease in turbidity of a standardized suspension of PHB granules The reaction mixture contained 100 mM Tris-Cl, pH 7.9, 1 mM $CaCl_2$, 180 µg of PHB, and 100 µL of culture supernatant or SNC (no enzyme control) in a final volume of 1.0 mL. The reaction mixture was prepared in 12 x 75 mm glass tubes without culture supernatant and preincubated for 5 min at 37º C. Culture supernatant was added to initiate the reaction, and the A_{650} of the mixture was determined 90 min after enzyme addition. The ΔA_{650} was calculated by subtracting the A_{650} of the sample from that of a no enzyme control. PHB depolymerase activity was calculated from the ΔA_{650} per min using an apparent extinction coefficient of 0.00370 A_{650}/µg. A unit of PHA depolymerase activity was defined as the amount of enzyme that degraded 1 µg of PHA per min at 37 ºC

3-HB Assay: 3-HB concentration was determined as R,S 3-HB concentration by a modification of the method of Buttery, Chamberlain, and Pannall [10]. A master mix was prepared, containing 90.9 mM Tris-Cl, pH 9.5, 356 µg/mL iodonitroetrazolium chloride, 222 µg/mL NAD^+, and 22.2 µg/mL phenazine methosulfate. Samples or standards containing up to 10 mM 3HB were prepared in water to a final volume of 25 µL, and 550 µL of the master mix was added to each. To each reaction was then added 5 µL of 3-Hydroxybutyrate dehydrogenase (3HBD) (from Rhodobacter sphaeroides, catalog # 10127833001, Roche, Basel, Switzerland), followed by incubation in the dark at room temperature for 15 min. After 15 min, 1.0 mL of 0.1 M HCl was added to each tube, while still keeping the tube in the dark. Optical density was determined at 510 nm and 3-HB concentrations calculated from a standard curve performed.

Spore Count Assay: Spore counts were determined by plating dilutions of heat-treated and sonicated sample cell pellets on trypticase soy agar (TSA). This treatment is thought to selectively kill vegetative cells while allowing spores to survive [6]. First, 1.4 mL of sterile deionized water was added to each pellet. The pellets were heated at 60 °C for 10 min and were then sonicated for 2 min at 35% power in a Fisher Sonic Dismembrator Model 300 (ThermoFisher Scientific, Waltham, MA). Dilutions of the treated cells were prepared by adding 90 µL of sterile deionized water to all wells of a sterile microtiter dish. 10 µL of sample pellet was added to the first well and mixed with pumping. 10 µL of sample from that well was transferred to an adjacent well as a dilution factor. Pellets were diluted thusly from 10^{-1} to 10^{-7}. TSA plates were prepared by drawing seven equal circles of 2.54 cm diameter and plating 10 µL of each dilution in its respective circle. The plates were incubated at 30 °C for 24 hrs, the colonies were counted, and the viable spore count was calculated.

Results and Discussion:

In this study, a growth curve analysis was performed to allow the cells to grow to mid-logarithmic phase to ensure the cells had experienced adequate growth for glycogen production under starvation conditions. In addition, the amount of cells for both species entering the sampling phase were standardized using wet weight. This method allows both the WT and *bld4* mutant to begin sampling with the same amount of cells, which allows the results to be interpreted equally.

Previous studies using the medium replacement technique showed the *bld4* mutant of *Streptomyces* sp. SFB5A to be deficient in glycogen production compared to the WT [7]. The goal of the present study was to see if this effect would hold true at lower inoculum levels. However, it has been noticed that starter cultures for experiments inoculated with low cell numbers (as with colonies from an agar plate), the mutant grows considerably more slowly than the WT, which might skew results. If it was again found that the mutant was deficient in glycogen production, it was essential to ensure that it was not simply due to its cells having to grow and catch up to the WT. Thus, to prepare inocula for our experiments, both strains were grown to mid logarithmic phase as determined by growth curve analyses, and the adjusted the cell wet weights of both strains to be the same. Accordingly, the inocula for both strains were in the same physiological state and equal in cell mass.

In the present study, using the lower inoculum method, two separate trials with the WT and *bld4* mutant were run. The glycogen accumulation assay demonstrated similar results to previous studies. In both trials, as seen in Figure 1, the WT exhibited a slight shoulder from day two to day three, which may indicate phase one glycogen. In both trials, the *bld4* mutant exhibited a gradually increasing, fairly smooth curve. As with previous studies [7], the WT accumulated more glycogen that the *bld4* mutant from 2 to 5 days. However, in trial two, *bld4* accumulated more glycogen that the WT, at least over the first 4 days. Further trials will be needed to determine whether the abnormalities found in *bld4* mutant in trial two were due to error. It is possible that prolonged use of anthrone in the same cuvette resulted in differing absorption readings. It is also possible that the tungsten bulb of the absorbance reader, Beckman DU 640, was malfunctioning.

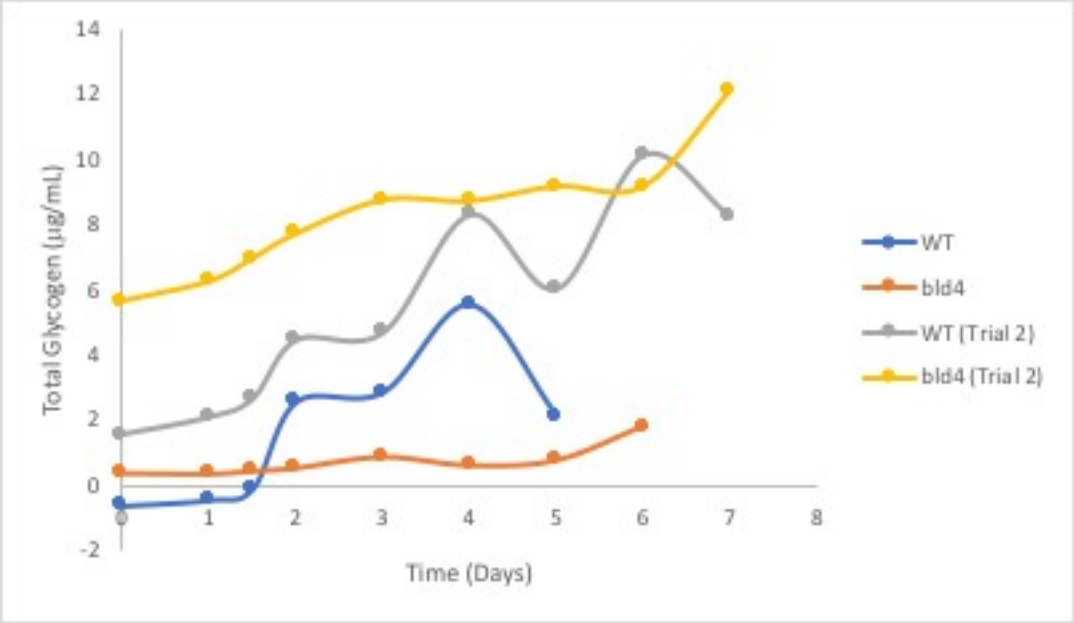

Figure 1. Glycogen accumulation during growth of *Streptomyces* sp. SFB 5A WT and the *bld4* mutant in SNC + 3HB. Assays were performed at the indicated time intervals. Data points are averages of four replicates.

The cellular protein assay produced inconsistent and inexplicable results, as shown in Figure 2. In both trials, the assay produced negative results that were unable to be interpreted, which means that the absorbances of the samples were below the readings of the blanks. These results may be due to not enough sample being used in the assay. Future studies should be designed to address this issue. This assay could be improved by using more sample in the microtiter dish and eliminating the use of deionized water.

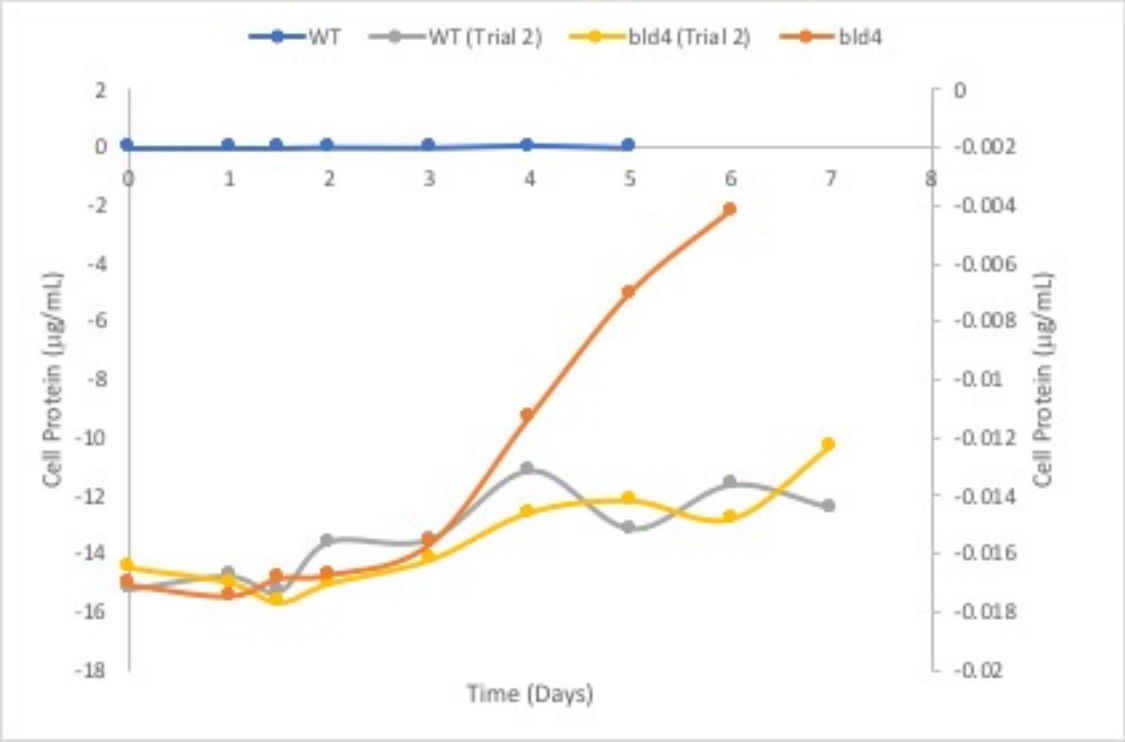

Figure 2. Cell protein during growth of *Streptomyces* sp. SFB 5A WT and the *bld4* mutant in SNC + 3HB. Assays were performed at the indicated time intervals. Data points are averages of four replicates.

The PHA depolymerase assay did not yield any activity for either strain at of the times sampled (data not shown). This is most likely due to enzyme degradation due to excess heat. These samples were kept on ice at all times, but the thawing process, which included warming the tubes using body heat, may have resulted in the proteases created by this species degrading the enzyme. In addition to the thawing process, the supernatant had to remain in the liquid state for at least five minutes in order for replicate assays to be performed. This could possibly be modified to using a method that allows for all samples to enter the assay at once to reduce the chance of protease activity. Future studies should be conducted in a manner in which the supernatant does not become warm enough for the depolymerase enzyme to degrade, and assays should be conducted soon after sample collection. Also, protease inhibitors might be added to the samples upon collection.

Previous studies using the medium replacement technique showed that the WT and *bld4* metabolized 3-HB at approximately equal rates [7]. However, the 3-HB degradation assay produced conflicting results, as shown in Figure 3. In trial one, the WT degraded half of the original 3-HB in three days, while the *bld4* mutant degraded half of the 3-HB in six days. Although, trial 1 must be rejected because the sample supernatants were not left on ice and the 3-HB was probably degraded by residual cells in the culture supernatants. Results of trial 2 suggested that the WT initially degraded 3-HB faster than *bld4* over the first 3 days. However, this analysis was complicated by the fact that the WT levels increased between days 3 and 4. This would indicate secretion of 3-HB from the cells, which is unlikely. Future studies should replicate this assay to determine if the differences found in trial 2 were due to error. Also, this assay may need to be modified, so that the supernatant is not as susceptible to protease activity.

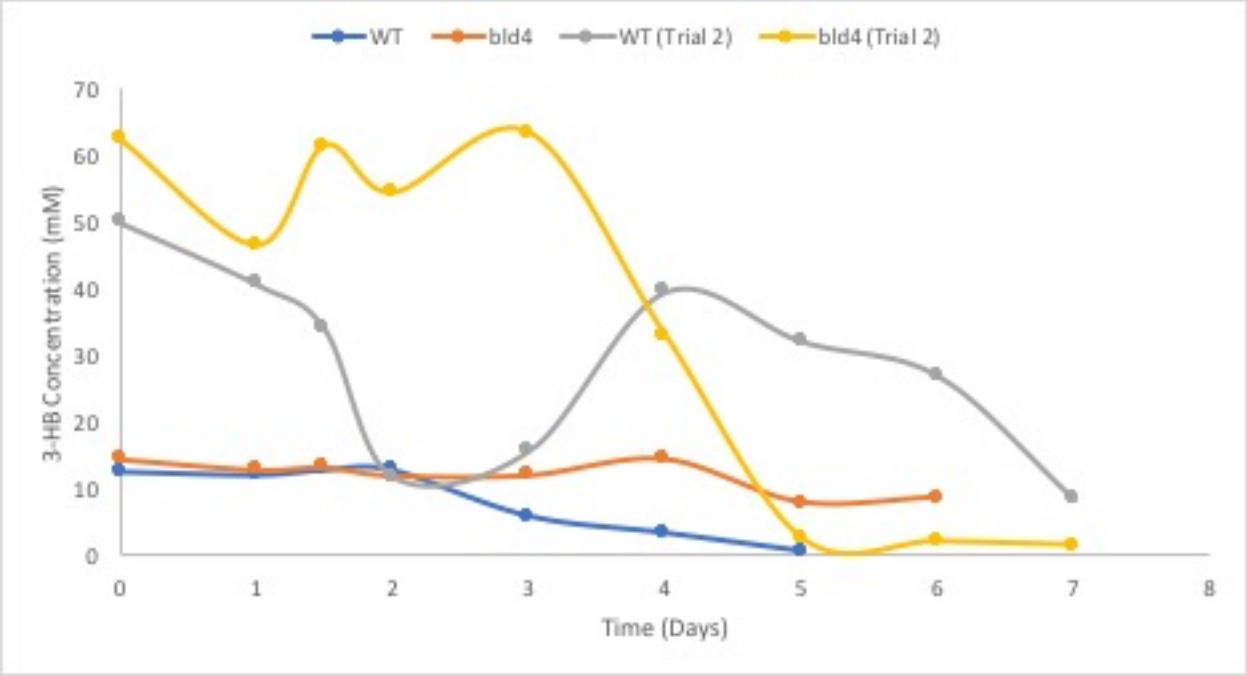

Figure 3. 3-hydroxybutyrate degradation during growth of *Streptomyces* sp. SFB 5A WT and the *bld4* mutant in SNC + 3HB. Assays were performed on samples removed at the indicated time intervals. Data points are averages of four replicates.

The spore production assay only produced interpretable results in trial one, since trial two experienced systematic error due to contamination. In trial one, the WT sporulated quickly, as shown in Figure 4, but no spores were detected after day three. The *bld4* mutant began producing spores at day four. The lack of spore production by the WT after day four may have been due to systematic error, such as a poor dilution. Future studies should replicate this procedure to determine spore behavior of both species with certainty.

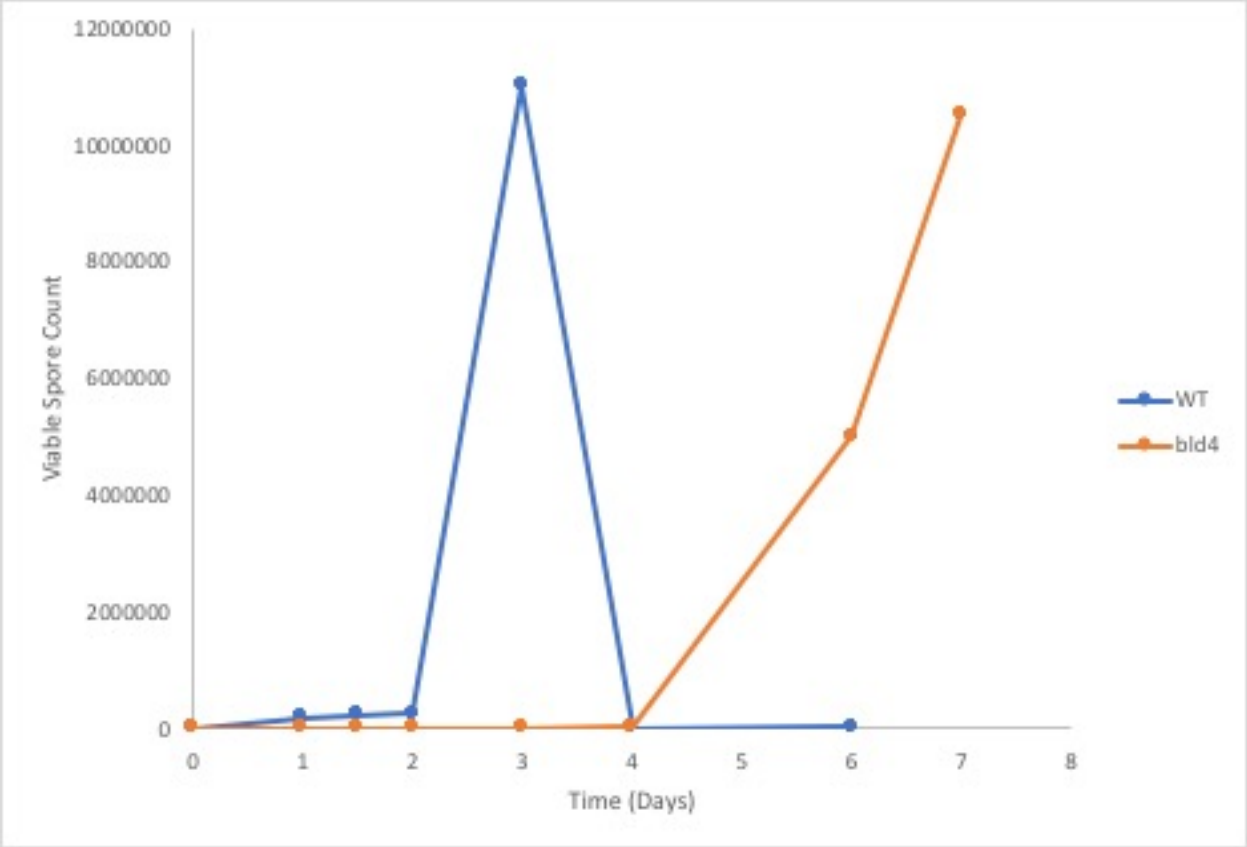

Figure 4. Spore production during growth of *Streptomyces* sp. SFB 5A WT and the bld4 mutant in SNC + 3HB. Assays were performed at the indicated time intervals.

During this project, two separate trials with the WT and the *bld4* mutant were performed. Based on previous research [7], it was hypothesized that the WT would accumulate glycogen more readily than the *bld4* mutant. If trial two's results are rejected due to error, a possible faulty cuvette or light source, then this hypothesis is supported. It was hypothesized that the WT would degrade 3-HB more efficiently and quickly than the *bld4* mutant. Since trial one has to be rejected due to the sample cell supernatant not being left on ice and the *bld4* mutant degraded 3-HB at a faster rate than the WT in trial two, this hypothesis was not supported. It was hypothesized that the *bld4* mutant would not produce spores; however, trial one suggests that the *bld4* mutant does produce spores two days behind the WT. Since no analyzable results were gathered from the cellular protein or PHA depolymerase assays, there respective hypotheses could not be evaluated.

In the future, this project should be continued by 1) replicating this study for validity; 2) comparing this low inoculum study to a high inoculum study directly; and 3) searching the literature for a stain to detect spores.

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