Trehalose as a Carbohydrate Source for the Culture and Stability of the Phase I Variant of *Photorhabdus luminescens*

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Abstract

Photorhabdus luminescens is a Gram-negative, bioluminescent, pigment producing enteric bacterium that is pathogenic to insects. This bacterium has the ability to undergo phase variation. The phase I variant exists as a mutualistic symbiont in the digestive tract of the nematode, *Heterorhabditis bacteriophora*, and plays a critical role in the life-cycle of this commercially-reared beneficial entomopathogenic nematode. Only phase I cells support development of the nematode. It is critical to understand the stability requirements of this bacterial variant. The bacterium receives its requirements (i.e. carbohydrates) from the hemolymph of the larval insect host. The sugar trehalose, an α-1,1 linked non-reducing disaccharide of glucose, is important in the physiology of microbes, insects and nematodes. Trehalose has been shown to be the most abundant sugar found within hemolymph. Acting as a physical and chemical protectant, trehalose has been implicated with thermal stress, dehydration and osmotic protection. It is also a store of glucose for glycolysis. We propose that trehalose will best support, in vitro, the stability of the phenotypic traits of the phase I variant. The traits monitored in this work are (1) bioluminescence (2) the production of the red anthroquinone–derived pigment and (3) culture pH. Shake flask cultures of *P. luminescens* were grown at 25°C for up to 7 days in basal media supplemented with different carbohydrates with increasing concentrations of 0.1%, 0.5% and 1.0% (w/v). The carbohydrates used in this study include: glucose (GLU), fructose (FRU), trehalose (TRE). Initial and final culture pH was recorded. The pH-dependent pigmentation was visually compared to a standard pigmentation scale. Relative bioluminescence (RLUs) was measured with a Turner BioSystems Modulus® luminometer. Serial dilutions were performed to determine phase I cell density. For the sugars tested, relative luminosity is dependent upon the carbohydrate used and incubation time. For GLU, the maximum RLU (4.36 x 10^{5}) was reached after 22 hrs, fell to 2.76 x 10^{5} after 27 hrs and rapidly decreased to 9.73 x 10^{4} at 43 hrs. For FRU, the maximum RLU (3.20 x 10^{5}) was reached at 43 hrs, and then drastically decreased to 6.29 x 10^{4} at 86 hrs. For TRE, the maximum RLU (1.87 x 10^{5}) was reached at 22 hrs and gradually decreased to 7.34 x 10^{4} after 146 hrs. TRE best maintained luminescence over time. The final pigmentation of the TRE media surpassed that of GLU, FRU and controls, indicating the highest density of phase 1 cells. Increased TRE concentrations in cultures were found to maintain stable pH between 8.2 - 8.3, explaining differences in acid production and pigmentation of non-TRE cultures. This data supports the hypothesis that *P. luminescens* cultured in basal media supplemented with trehalose best maintains the phenotypic traits of phase 1 variants.

Introduction

Trehalose is a naturally occurring disaccharide that is formed by an α-1,1 glucosidic bond (Figure 1). This bonding pattern makes trehalose very resistant to acid hydrolysis and stable at high temperatures even under autoclaving conditions. This disaccharide can be synthesized and utilized by many organisms including bacteria, fungi, plants, mammals and many invertebrate animals (e.g. nematodes and insects). In insects, trehalose has been found as the most abundant sugar within the insect hemolymph where it is used as a quick energy source for the insect. Trehalose catabolism is accomplished by the enzyme, trehalase. In insects, trehalase hydrolyzes the α-1,1 bond transforming trehalose into two units of glucose (Figure 2) that can be used for high energy activities such as "jumping" and "flying". *Photorhabdus luminescens* is a Gram-negative, enteric bacterium that is pathogenic to most insects and lives in a symbiotic relationship with the entomopathogenic nematode, *Heterorhabditis bacteriophora*. This bacterium has the ability to phase shift meaning that it is able to shift between two different variations (e.g. phase I and phase II). It is known that the phase I variant of this bacterium is able to support the life cycle of the nematode. However, under laboratory conditions it is difficult to maintain the phase I variant because the bacteria have a natural tendency to shift into the phase II variant. Therefore the purpose of this research is to determine a suitable carbohydrate source that is found within the insect that will allow for a longer maintenance period of the phase I variant.

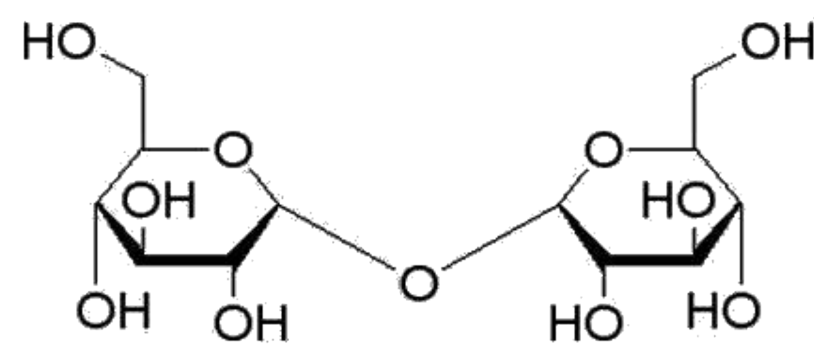


Figure 1. Molecular structure of trehalose

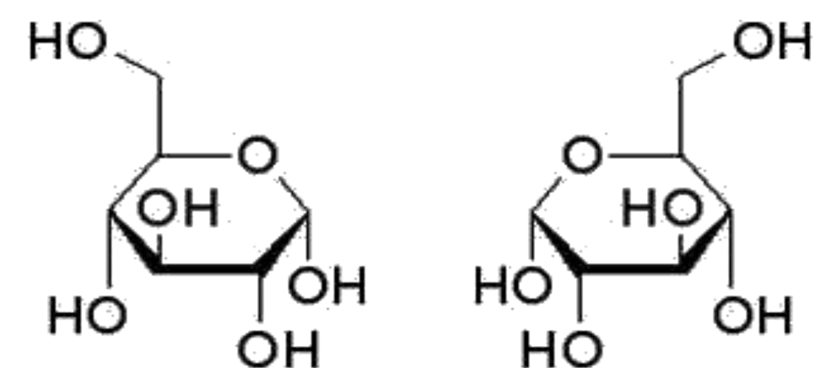


Figure 2. Trehalase production of two glucose molecules

Materials & Methods

The phase I variant of *P. luminescens* was cultured in a 2 liter fermentation vessel grown at 28°C for 2 days (Figure 3). Basal media consisted of 0.3% of beef extract and 0.5% of digested gelatin per liter of water (w/v). Shake flasks of basal media, except for the control, was supplemented with increasing concentrations of filter-sterilized carbohydrate solutions. Carbohydrates used were glucose, fructose and trehalose. Concentrations of 0.1%, 0.5% and 1.0% (v/v) were used per carbohydrate. Shake flasks containing the supplemented media were inoculated with the phase I variant at a 3% concentration, cultured at room temperature (25°C) and agitated on a platform shaker at 150 RPM (Figure 4). Just after thorough mixing (3 min), bioluminescence readings were recorded and time was started. Relative luminescence units were measured and quantified with the Modulus luminometer set for a reading cycle of 10 seconds. Shake flask samples were taken throughout the 7 day period at random intervals of bacterial growth. Shake flask samples from all concentrations and sugars were respectively collected at the same time. At sampling, pigmentation was also recorded and scored using a created standard pigmentation scale.

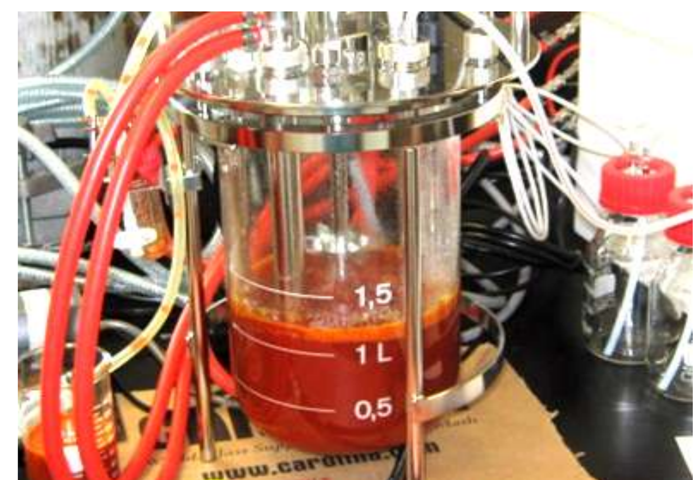
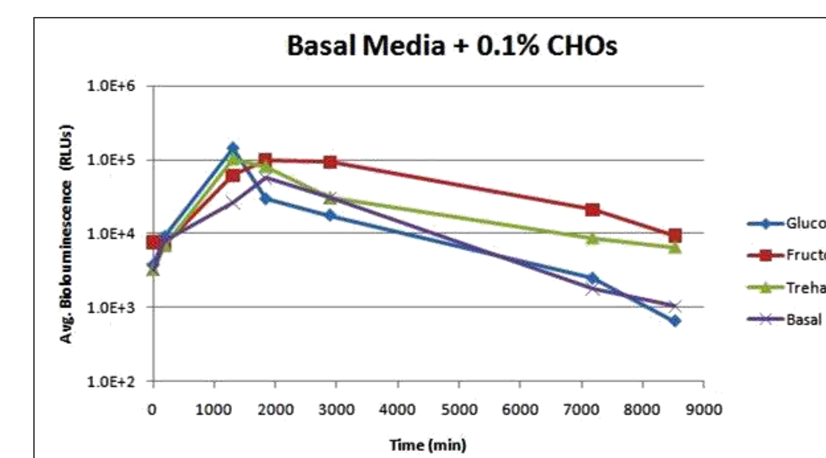


Figure 3. Culturing of the phase I variant

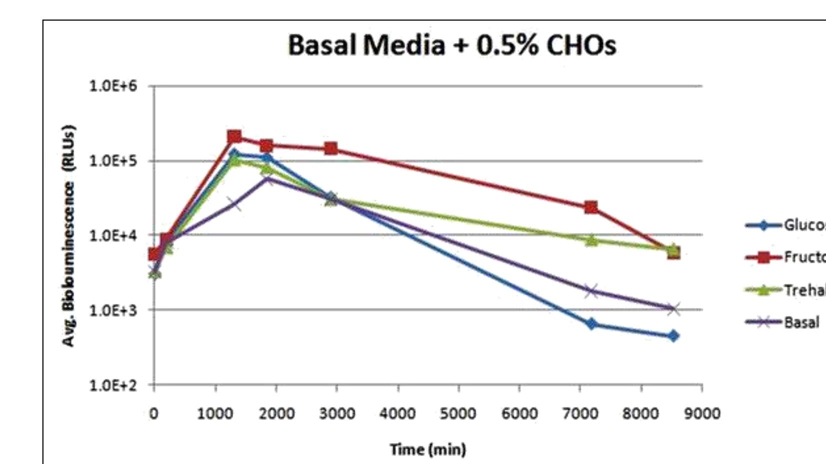
Figure 4. Inoculated shake flasks

Results

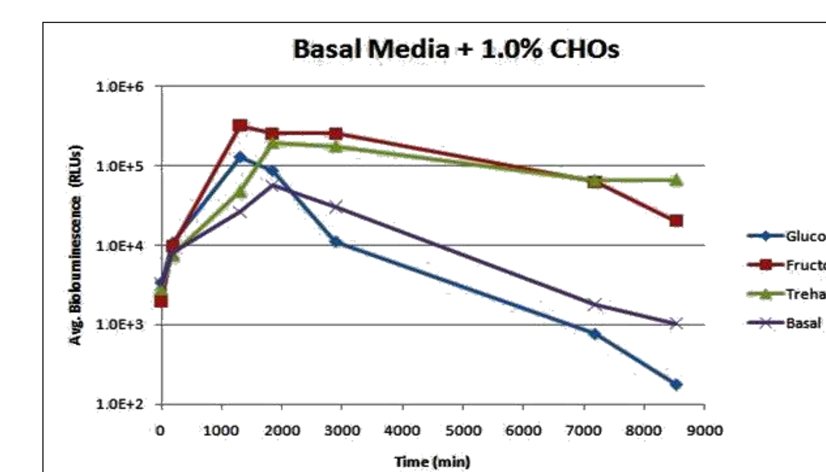
Average relative luminescence units (RLUs) were measured and graphed as a function of culture age. It is hypothesized that 1.0% trehalose can maintain the phase stability and bioluminescence of *P. luminescens*.



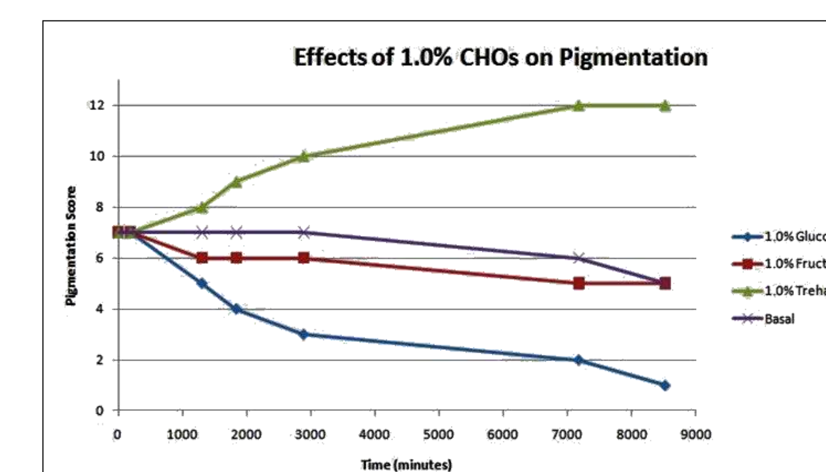
Graph 1. Basal media with 0.1% carbohydrates



Graph 2. Basal media with 0.5% carbohydrates



Graph 3. Basal media with 1.0% carbohydrates



Graph 4. Effect of basal media with 1.0% carbohydrates on pigmentation production

Figure 5. Pigmentation of Basal media with 1.0% CHOs From left to right: TRE, FRU & GLU.

Graph 1 depicts basal media with 0.1% CHOs. With all CHOs luminescence increased up to 1.0 x 10^{5} around 1,000 minutes with glucose declining rapidly compared to the other CHOs and the control. With 0.1% FRU, declined slightly and dropped steeply between 7,000 and 8,000 minutes. However, TRE shows a gradual decline from the initial decline around 2,000 minutes and continued to gradually decline slightly at 8,500 minutes and could potentially be maintained at this rate.

Graph 2 shows basal media with 0.5% CHOs. With all CHOs, luminescence peaks around 1.0 x 10^{5} at about 1,000 minutes. 0.5% FRU was able to still maintain a high luminescence up to around 3,000 minutes but the rate of decline is higher than that of TRE. Again TRE shows a slight gradual decline whereas GLU falls below the control.

Graph 3 demonstrates that at a 1.0% concentration, TRE is able to maintain an average luminescence of about 1.0 x 10^{5} over an extended amount of time whereas FRU, GLU and the control are continuously decreasing .

Graph 4 shows basal media with 1.0% CHOs. Notice the pigmentation of the cultures. At the ending of the experiment TRE had a pigmentation score of 12, FRU and the control with a score of 5 and GLU with a score of 1. Figure 5 shows a photograph of the three basal medias with the corresponding 1.0% CHOs.

Discussion

Trehalose, the "blood sugar" of insects does seem to maintain the stability, pigmentation and bioluminescence of the culture as a function of culture age. As the concentration of trehalose is increased, all of the phase I variant phenotypic traits are enhanced and maintained.

It may be plausible to say that a concentration of 2.0% TRE may indeed maintain the stability, pigmentation and bioluminescence of the phase I variant of P. luminescens for longer periods of time that is required by the nematodes to develop and reproduce *in vivo* and *in vitro*.

Acknowledgements

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