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Degradation of Cellobiose via *Bacillus stearothermophilus*

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ABSTRACT

Due to rising fuel costs and worries of depleting fossil fuel reserves, the need for alternate fuel has arisen. Through this crisis biofuel has emerged as a front runner as a renewable resource. Biofuel production is the process of creating the fuel Ethanol with the usage of microorganisms and plant material. Originally biofuel has been created by using materials such as corn, which is a valuable resource for human consumption. But it has been found that certain bacteria are very capable of degrading biomass, that would otherwise be unusable, into sugars such as sucrose and glucose which can be later converted to ethanol. Biofuels are created by fermenting plants rich in starch, but some bacteria have the innate ability to degrade cellulose which is found in all plants. In this study I show the usage of the common bacteria *Bacillus stearothermophilus* as a possible way of converting otherwise non-usable plant matter into a vessel for ethanol production. The bacteria were allowed to incubate for 24 hours, and then successive measurements were taken every two hours afterward using a VAPRO vapor pressure osmometer to analyze total concentration of dissolved particles in solution. By running a repeated measure ANOVA and paired T-test it was clear to see that there were significant difference between concentrations and time elapsed.

Keywords: Biofuel, Cellulosic Ethanol, Cellulase, *Bacillus stearothermophilus*

INTRODUCTION

In recent years, the problems of depleting global oil reserves and increased climatic change has led to the increasing interest of alternate energy (Rao PR, N Dufour, and J Swana. 2011). One such source of this energy is ethanol, which is produced from biomass such as corn. Ethanol is a suitable substitute for petroleum, but it uses valuable resources for its production which can be used for human consumption.

This dispute has created a food versus fuel dilemma, driving the prices of consumable human products higher and forcing researchers to look for a way to use cheap non-usable biomass for ethanol production. These cheap non-consumable biomasses such as agricultural wastes or industrial byproducts pose a good source of lignocellulosic biomass, but converting the wastes into usable energy sources has been a major problem of the second generation biofuel production. Over the past few decades researchers have probed into the possibilities of using bacteria to degrade this waste into usable energy source. At the forefront of this bacterial research are many types of thermophilic bacteria with optimal growth temperatures over 60 degrees Celsius. (Chang, Shuo Yao) Bacteria such as *Bacillus stearothermophilus* show promise in degrading material rich in cellulose into usable sugars like glucose for fermentation.

Bacillus stearothermophilus is a gram positive, rod shaped bacterium that is easily obtained from the environment. This bacterial species is thermophilic with an optimal growth temperature of 60 degrees Celsius. At these high temperatures it is very challenging for other bacteria to survive, or actively grow and reproduce which means that this bacterium is a good choice when it comes to fuel production. *B. stearothermophilus* contains the enzyme cellulase which can degrade the cellulose in plant material to usable glucose for the fermentation of ethanol. This enzyme attacks the crystalline structure of the cellulose molecules and hydrolyzes the 1, 4-beta-D-glycosidic linkages in cellulose. The structure of the beta link in cellulose makes it difficult to degrade and makes it a very strong organic structure.

The mechanism by which cellulase is degraded via *B. stearothermophilus* can be measured and therefore it can be calculated. My goal is to understand the rate at which the bacteria can degrade cellulosic material in solution.

MATERIALS AND METHODS

Bacteria for this experiment were obtained from Carolina Biological Supply. The bacteria were then inoculated in one 125mL Erlenmeyer flask containing 100mL of defined *B. stearothermophilus* broth to create a uniform broth to ensure roughly the same amount of bacteria would be transferred to each flask for the experiment. Two broths were made, the first not containing cellobiose, the second containing it. The process was exactly the same for constructing both broths minus the additive of cellobiose to one. The purpose of this was to see if the addition of cellobiose had a significant impact on concentration levels in the media.

Before inoculation of the broth a sterile sample was taken for baseline readings. To ensure optimal growth after inoculation the bacteria were stored at

60˚C.

The broth was made using the Difco Manuals' standard *B. stearothermophilus* broth. The broth was made by mixing 10g pancreatic digest of casein, 5g yeast extract, 2g K_2HPO_4, and 2.5g cellobiose. Cellobiose was chosen in this experiment due to the fact that cellulose does not readily dissolve into solution. The ingredients were mixed on a hot plate with a magnetic stir bar rotating at 100 revolutions per minute at a temperature of 90˚C for ten minutes. This insured that all the components of the media were fully dissolved into solution. After completely mixing the ingredients, the broth was poured into 10 125mL Erlenmeyer flasks and autoclaved at 121˚C for 15 minutes to ensure the sterility of the broth.

The broth and bacteria were then transferred to the Laminar Sterile hood for inoculation. This hood was used to ensure that there was no cross contamination from airborne bacteria. Sterile technique was used for inoculation of the flasks.

To promote growth, the cultured flasks were stored in an incubator at 60˚C for 24 hours for an initial incubation period. Since the bacteria have reached the peak of its growth phase after 24 hours, samples were taken from all ten flasks every 2 hours after the first day of incubation for an additional 10 hours. At this stage the bacteria has theoretically started utilizing cellulase.

The samples were taken using a 1000mL pipette and transferred into 1mL micro centrifuge tube, then put into a deep freeze set at -80˚C to promptly stop all cellular activity. After all sampling was complete the solution containing the broth and bacteria were centrifuged at 7000 rpm for ten minutes to compact dead cells at the bottom of the tube. After centrifugation was complete data was collected by using a VAPRO vapor pressure Osmometer. Results were measured in mmol/kg to determine the amount of particulate matter dissolved into solution.

RESULTS

The bacteria showed very clear signs of initial growth after the 24 hour incubation period, but after the first tests, results were unclear. The data could not be quantified in any clear manner as the test was contaminated by human error. The following tests proved to be efficient and show a positive correlation of assumed increase in glucose concentrations over the allotted test period in the broth, thus disproving the null hypothesis that there would be no change in concentration over time.

The initial samples of sterile media showed concentrations of 144.25±7 mmol/kg solution. The tests taken in the first 2 hour time slot after incubation showed a decrease in dissolved particulate matter. The tests showed concentrations of 95.11±3 mmol/kg. This initial drop in concentration was believed to be caused by the bacteria's initial growth and consumption of nutrients in the media. After 28 hours the concentrations of the solution had risen to 103.04±5mmol/kg, which showed that matter was being put back into solution as byproducts. After 30 hours the concentrations continued to rise to 115.71±3. At 32 hours concentrations were at 120.50±2 mmol/kg and after 34 hours the broth registered a final change in concentrations as levels reached 126.21±2 mmol/kg.

This data was analyzed using a paired T-test and repeated measures ANOVA test. All P values were shown at $P<0.0001$. To be certain that this was conclusive, α was adjusted from 0.05 to 0.002 to compensate. The results were still conclusive that there was a significant difference between the media and time intervals in the broth for all flasks.

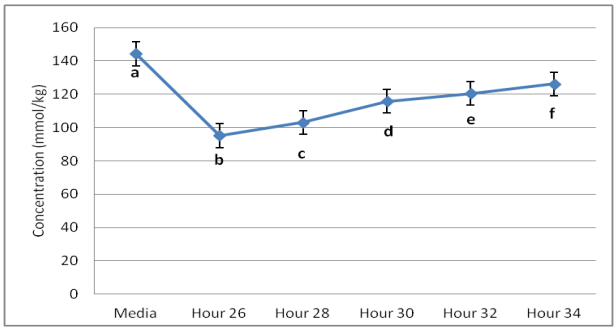

Figure 1: Depicts Media concentrations over time including standard error. Lowercase letters denote individuals of T-test with significant differences.

DISCUSSION

This research was proposed to try and understand maximum levels of cellobiose degradation. The results of this study show that after 24 hours of incubation, *B. stearothermophilus* could be capable of degrading cellobiose into usable sugars such as glucose. But after completing this research it was evident that different techniques could have been applied in analysis of the bacteria broth. Methods of utilizing gas chromatography after the experimentation period could have gave more information of what was within the broths composition.

As outlined by S Subramaniyan, P Prema, (2006) the bacterial strain shows possible signs and potential of degrading cellulose. But the substance in the media could not be determined and further testing would be needed for conclusive results.

Ideally this process used in this experiment could be applied to a controlled system, B. Hartley (1983), which could harvest the media when the bacterium has reached a maximum output of degraded cellulosic material. Then the material could be

processed and fermented into ethanol. With the utilization of thermophilic bacteria in the production of alternate fuels, the possible outcomes are substantial.

B. stearothermophilus could also possibly be genetically altered to maximize the output of cellulase to increase the yield percentage of monosaccharaides such as glucose. As of now strains of *B. stearothermophilus* have relativity low levels of cellulase activity (S Subramaniyan, P Prema 2006) Future research is being conducted on the usage of thermophilic bacteria for ethanol production. By removing the L-lactate production by selecting mutations in L-lactate dehydrogenase, mutant strains of *B. Stearothermophilus* are capable of producing ethanol in surprising high concentrations
(B. Hartley, 1987)

Further study is also needed to quantify the ability of *Bacillus stearothermophilus* and its possible mutant variations to degrade cellulose, and emphasis should be placed on the actual production of ethanol via thermophilic bacteria. The nature of these bacterial strains allow for ideal conditions of growth unhindered by other microbes competing for nutrients. Emphasis could also be place on cloning *B. stearothermophilus* to discover other applications in real world situations. (Hussain, AA, H Majeed, and A Nooria. 2011)

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