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ELICITATION OF POLYHYDROXYALKANOATES (PHA) FROM *BACILLUS SUBTILIS* IN FERMENTOR SYSTEM

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Abstract: This research investigates the elicitation of the *Bacillus subtilis* of its PHA content, the polyhydroxybutyrate (PHB), by applying pH gradients from its optimum conditions of 37℃ and pH of 6-8. Analyses of results were done by gravimetric analysis, and presence of PHB was verified by Gas Chromatography-Flame Ionization Detector. Results have shown that PHA was elicited only at ΔpH 2 and 12, having 17.86% and 25%, respectively at the instance of pH change. Furthermore, six hours after the application of stress, %PHB elicited for ΔpH of 2, 4, 10 and 12 had 41.67%, 25%, 46.15% and 50%, respectively. From these results, it was observed that PHA was instantly elicited for extreme ΔpH's. After the formation of spores, *B. subtilis* was able to resist chemical damages to elicit more PHA. However, no PHA was elicited for ΔpH=6. Overall, the pH responsible for the highest PHB elicitation was 12, having a yield of 0.68 mg PHB/mg dry cell weight.

Key Words: elicitation; polyhydroxyalkanoates, PHA; PHB; *Bacillus subtilis;* bioplastics;

1. INTRODUCTION

The world is now facing environmental crises brought about by pollution, global warming and other means of environmental destruction brought about by the modernization of the different activities of mankind. With all the damages the people have brought to the environment, it is also a responsibility of every individual to turn things around to achieve a sustainable environment for the next generations. Because of this, green technology was innovated, and one of which is the invention of bioplastics.

In the Philippines, it is necessary to address the plastics waste due to its high accumulation in dumpsites. In fact, a study conducted by the National Solid Waste Management Commission states that Metro Manila is said to contribute 6700 metric tons of solid wastes per day. From this waste, 45% come from kitchen wastes while the other 55% is brought about by the following: 16% from paper, 15% from plastics, 9% from glass and wood, and the others from other types of solid wastes. Also, the USAID-assisted Philippine Environmental Governance (EcoGov) conducted a project in ten of the municipalities in Metro Manila, and it shows that from the total solid wastes, 60% are biodegradable, 20% are recyclable, 18% are those that cannot be thrown into dumpsites while the other 2% are those considered as special wastes (The Philippines' Brown Environment, 2005).

Because of this high percentage of plastics in the solid wastes accumulated in Metro Manila per day, it really is recommendable for people to use bioplastics for their everyday routine such as doing groceries, or simply in using plastics for their trash. In terms of

production and degradability, bioplastics are more favorable over synthetic plastics. Bioplastics degrade after about 10 years unlike PP and PET which degrade for over a hundred of years (Gate2Biotech, 2007). Also, in terms of the manufacturing processes, it has been proven by Momani (2009) that bioplastics show a much reduced amount of CO_2 emissions compared to polyethylene.

Aside from bioplastics having a faster rate for decomposition, they are also renewable, easier to recycle, non-toxic in terms of bioplastics being decomposed and absorbed into the earth, and consume less than 50% of the energy needed in the production of non-biodegradable plastics (7 Advantages of Biodegradable Plastics, 2010). Although bioplastics seem to be more favorable in terms of its cost savings and degradability, not much has been studied with PHA production yet. In addition, most studies on PHA, however, focus on the extraction of the PHA, and not on its elicitation. If there are about elicitation, these are more focused on fungal elicitation or nutrient limiting, which still causes the disruption of the bacteria's cell wall, and continuous supply of PHA from the same culture is not achieved.

Because of this, the focus of this research is the elicitation of PHA from the bacteria by means of pH gradients. Upon the application of changes in the bacteria's media, these accumulate PHA in their cells and later elicit its PHA content to the media to sustain its viability. Thus, this research is about the determination of the pH at which the bacteria would release most of its PHA content by means of pH gradients.

2. METHODOLOGY

2.1 Experimental Flowchart

Cultures were kept in 250 ml Erlenmeyer flasks, were sent to the incubator shaker at 180 revolutions per minute, and were kept at a temperature of 37°C. Sampling was done every 12 hours to get the growth curve profile of the bacteria. Samples were subjected to cell concentration analysis, sugar consumption analysis, PHB content analysis and verification of PHB presence in the samples.

2.2 Experimental Set-Up

Figure 1 illustrates the 1L fermentor used in this experiment. The head plate includes a sampling port and an aeration port. The culture volume used was 600 ml. Samples were retrieved from the sampling port of the fermentor. To ensure that there is enough oxygen in the broth, the fermentor was pumped with air using a syringe connected to an air filter. The magnetic bar kept the broth agitated at 180 revolutions per minute and the broth was also maintained at 37°C. The system was maintained at these conditions by adjusting the stirring and heating knobs.

2.3 Retrieval of PHB Elicited

Sample was taken out from the media and was transferred to centrifuge vials for 45 minutes under 6000 revolutions per minute. 5 ml of the supernatant was added with 5 ml of chloroform to get the PHB elicited before and after the applications of pH. The samples were centrifuged at 3000 revolutions per minute for 20 minutes. Afterwards, with the use of a glass syringe, 1 ml of the chloroform layer was syringed out and was transferred to pre-weighed

sampling vials and the chloroform was allowed to evaporate under the fume hood. After drying, the mass of the PHB in the vials was weighed, and the mass of the vial was subtracted to get the mass of PHB alone. The mass of the PHB in the vial over the total amount of nutrient broth has given the concentration of the PHB elicited.

2.4 Extraction of Total PHB

The supernatant of the centrifuged sample was separated and the cell mass. The dry cell mass was added with 5 ml of 6% Sodium hypochlorite solution and 5 ml of chloroform. The mixture was agitated for 3 hours at 250 revolutions per minute and a temperature of 37°C. Then, the mixture was centrifuged at 3000 revolutions per minute for 20 minutes and 1 ml of the heaviest layer was taken out and was transferred to pre-weighed sampling vials. The chloroform was allowed to evaporate and the mass remained in the vial was the PHB. This was then weighed and the difference of this mass and the mass of the vial would give the mass of the PHB.

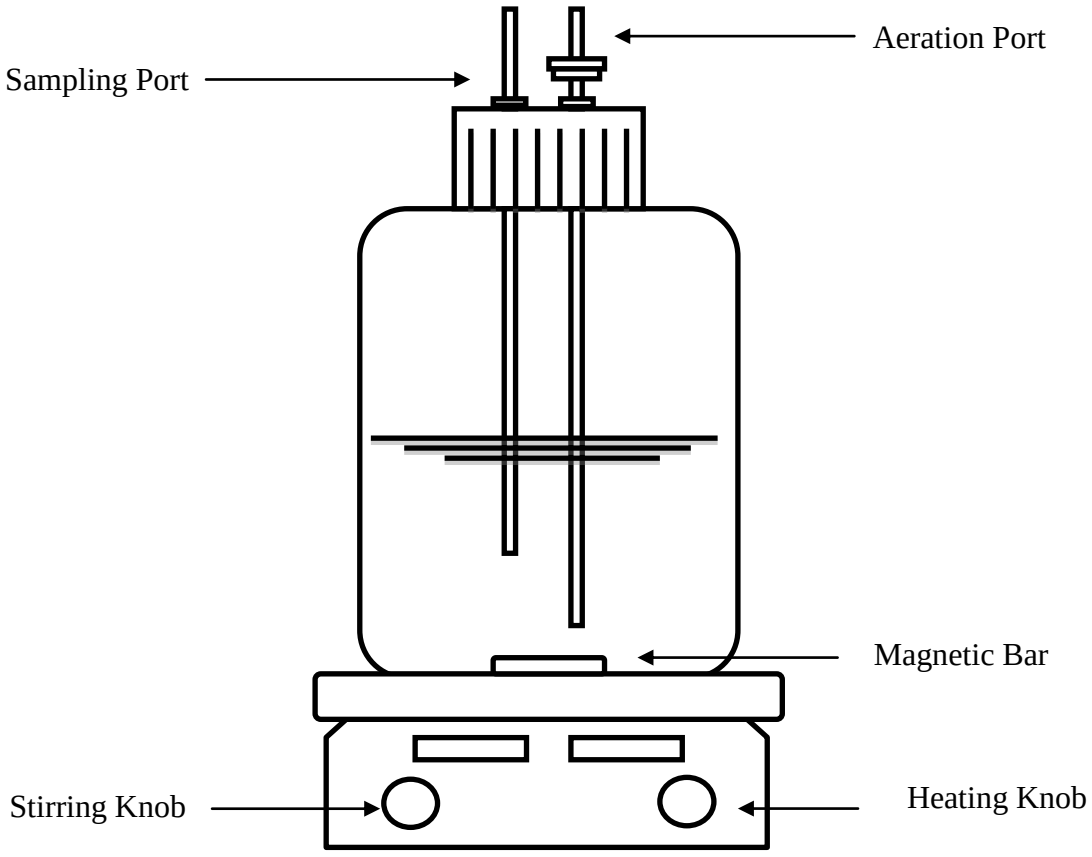

Figure 1 Fermentor Set-up

2.5 Analysis of PHB by GC-FID

Hydrochloric propanolysis method optimized by Riis & Mai (1988) was used before the GC analysis. After obtaining of about 40 mg of dry cell mass in 10 ml test tube vials, 2-

ml volume of Trichloroethylene, 2ml of acidified reagent and 200 microliter of 2 grams benzoic acid in 50 ml propanol, called the internal standard solution, was added. The mixture was kept in an oven at 100℃ for 2 hours. 4ml of distilled water was then added after cooling to room temperature. The resulting sample was shaken for 30 seconds then allowed to separate into two phases. The heavier phase (Trichloroethylene-propanol) was obtained by using a glass syringe and later injected into the GC (Riis & Mai, 1988). For the analysis, Elite-5MS column was used in a HP 5890 Gas Chromatography-Flame Ionization Detector (Hewlett-Packard, Avondale, PA, USA) provided by the Chemistry Department of De La Salle University, Manila. The isothermal temperature was 100°C while the carrier gas used was Helium, 2ml/min. The injection volume of the sample was 0.6 microliters (Riis & Mai, 1988).

3. RESULTS AND DISCUSSION

3.1 Polyhydroxyalkanoate Analysis

Figure 2 illustrates the relationship of the cell concentration and the PHB accumulation. The figure had an almost constant concentration with respect to the time, having a concentration of 0.0019 mg/mL on the 24.5th hour. The cells then started its exponential growth at 48.5th hour and still, PHB concentration was found to be 0.0015 mg/mL, which is nearly constant. As seen from the said figure, there was a very slight increase in the amount of the PHB, from 0.0015 to 0.0019 mg/ml in the cells, and became constant at 0.0015 mg/ml during the exponential phase. Minimal accumulation of PHB was observed since there was no stress subjected to the system. The work from Yüksekdag, et al, (2003) also had similar results with having very minimal increase in PHB concentration as growth increases, and decreased during its 48th hour of growth.

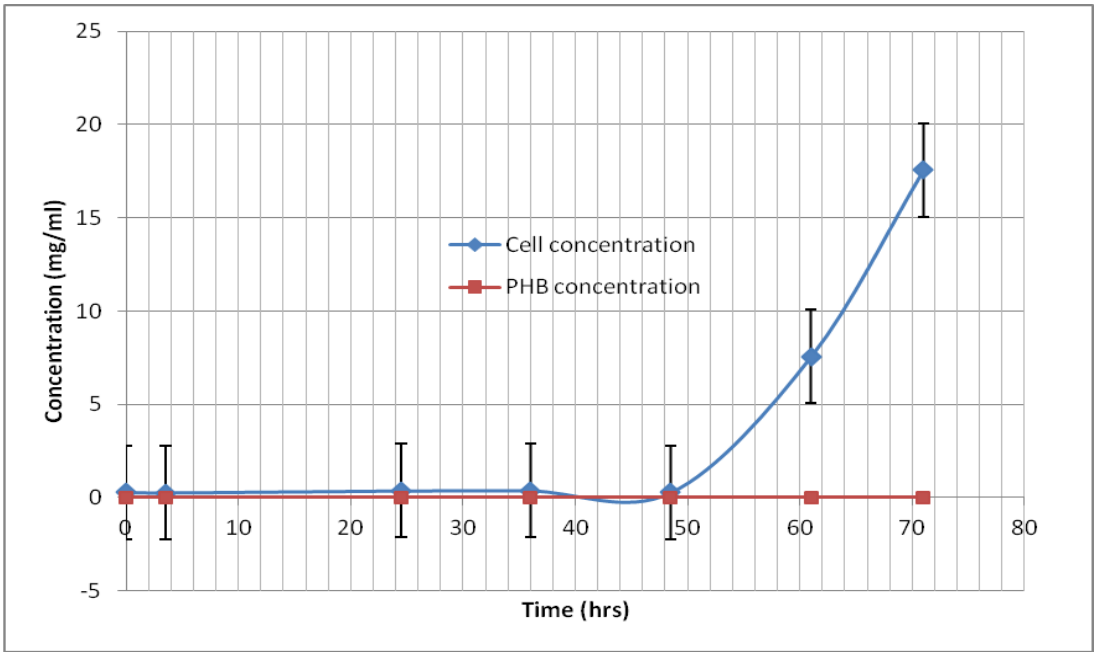

Figure 2. Correlation of PHB Concentration and Cell Concentration

3.2 PHB Analysis at pH Gradients

From Figure 3, it can be observed that an addition of pH gradient had increased the total PHB concentration. The PHB that had the highest increment after subjecting the culture to pH gradient was pH 12. This could be best explained from the study of Wilks, et al. (2008) on the acid and base stress transcriptomic responses in *Bacillus subtilis*, that having a high external pH environment favored catabolism-generating acids which would lead to the upregulation of the acid producing enzymes and the H^+ antiporters.

The yield of PHB was also calculated using the ratio of the dry cell weight concentration and the PHB concentration. The results were tabulated and shown in Figure 3.

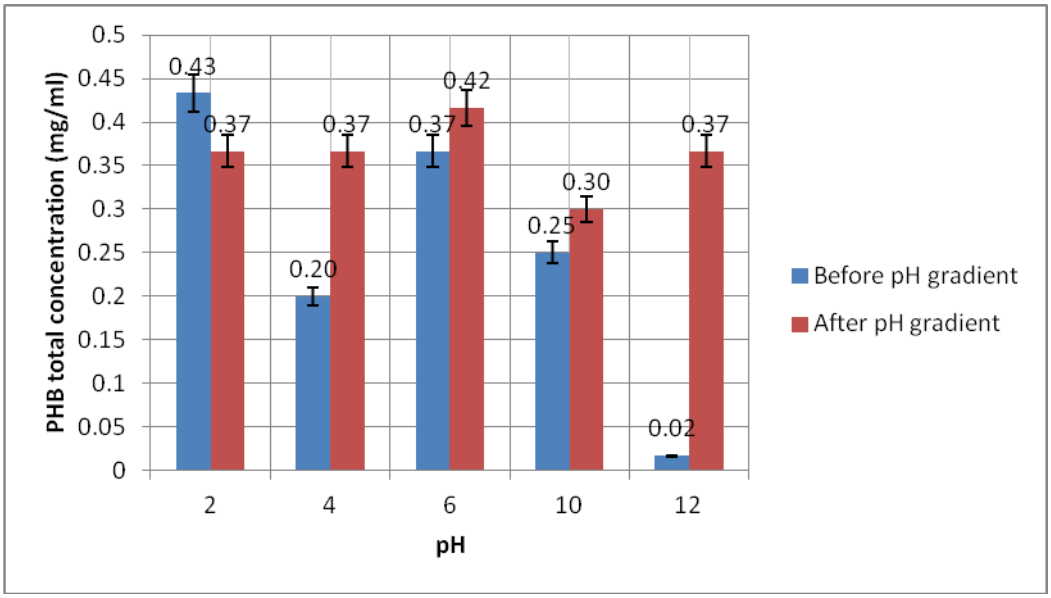

Figure 3. Total PHB Concentration in pH Gradients of pH 2, pH 4, pH 6, pH 10 & pH 12.

As observed in Figure 4, the yield of PHB was observed to have the highest change on the change from pH 8 to pH 12. This would also signify that there would be an increase in PHB yield in a very basic external environment for *Bacillus subtilis*, which can be supported by the theory and results from Wilks, et al. (2008) that high pH environments upregulates acid generating enzymes, which could possibly induce production of 3-(R)-hydroxybutyric acids. Also, increase in the yield at lower pH may be caused by the requirement need of an H^+ ion on the biosynthesis of PHB when using the NADPH dependent acetoacetyl-CoA reductase.

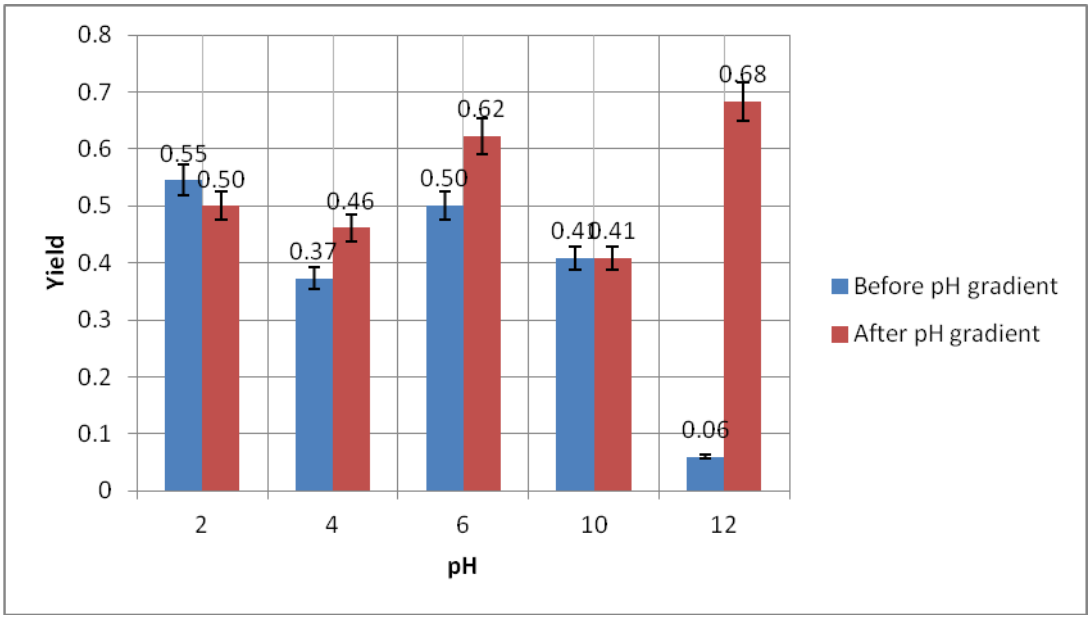

Figure 4. Yield of PHB with Respect to the Dry Cell Weight Concentration in pH 2, pH 4, pH 6, pH 10 & pH 12 Before and After pH Gradient.

The concentration of PHB elicited to the environment was also obtained to calculate the %PHB elicited to the external environment of the cells. The results were averaged and presented on Figure 5.

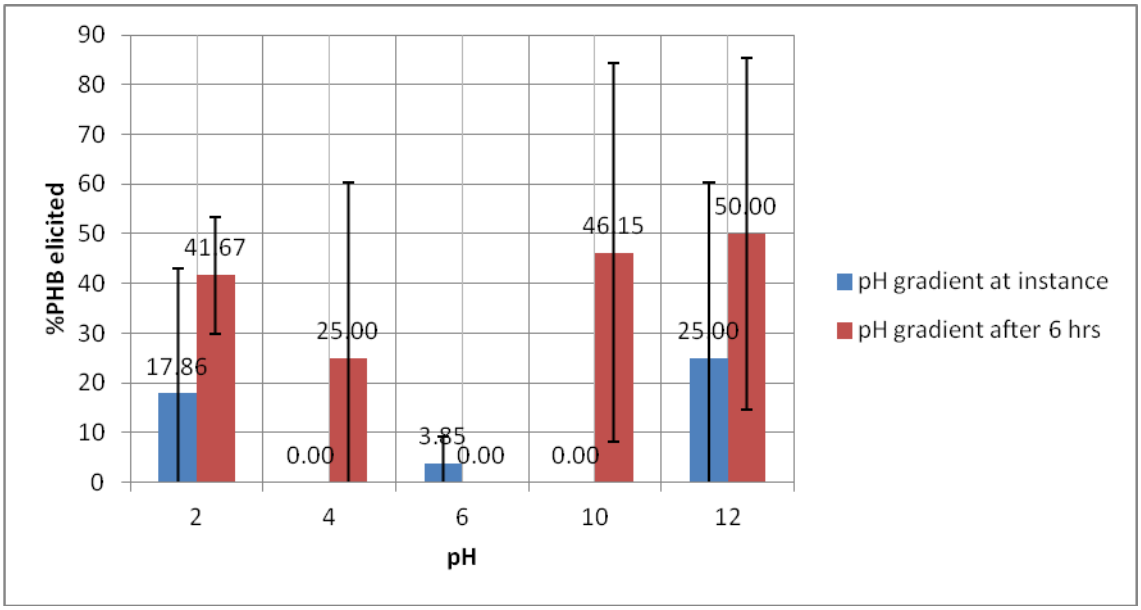

Figure 5. Percent PHB Elicited in pH 2, pH 4, pH 6, pH 10 & pH 12.

From Figure 5, it can be deduced that at pH 2 and 12, which were considered to be the extremes, the bacterium elicited PHB to the media. This signifies that more PHB is elicited to the media at a more acidic or more basic environment. The pH applied would possibly induce acid-generating enzymes that would trigger PHB depolymerase to act on PHB, which may

catalyze into smaller units of polymer that would be elicited through the cell wall. The lowest pH may have resulted in the lysis of cells and then the release of accumulated PHB into the media. Also, comparing the two sets of bar graphs in Figure 5, more PHB was elicited after 6 hours from the time of application of pH change. PHB was elicited instantly at the extreme pH of 2 and 12, while at gradients 4, 6 and 10, there were no significant changes in the PHB elicited from the cells. However, it was found that after 6 hours, more PHB was elicited to the media. This indicates that the bacterium underwent sporulation at the instant when pH change was applied, and after 6 hours, the bacterium has probably adjusted to the pH change by eliciting most of its PHB content to the media.

4. CONCLUSIONS

With the use of GC-FID application of pH changes, it was observed that at pH of 12, maximum PHB was retrieved. Also, upon the addition of HCl into the system, it was observed that the bacteria has stopped its growth, unlike that of basic medium whereas 2 weeks after the application of the pH change, the bacterium was able to return the pH of its media to around 6-8, which is almost that of its optimum pH. The application of pH gradient to the culture was found to have its highest increase in yield to be 0.68 g PHB/ g dry cell mass in pH 12 from 0.06. The highest PHB elicited in the culture was found to be 50% of the total PHB accumulated after changing the pH at 6 hours.

5. REFERENCES

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