Isolation, Biochemical Characterization and Optimization of Polyhydroxyalkanoates (PHA) Producing Bacteria

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Abstract: The environmental problems brought about by the overproduction and accumulation of plastic waste has brought interest to the development of bioplastics. Bioplastics are plastics made from renewable sources like oil, starch and even microbiota. Polyhydroxyalkanoates, or simply PHA, is a polyester produced by bacteria and is a promising material for plastic production. PHA is produced by bacteria as a stress response when the environment has limited supply of essential nutrients and abundant supply of carbon. Environments with limited amount of nutrients provide an appropriate condition for PHA production. Water samples were obtained from selected sites in Batangas City namely Tingga Falls and Boracads beach. PHA producing bacteria were isolated from Boracads Beach. The isolates were identified to be Bacillus subtilis. Several parameters like temperature, pH, carbon-nitrogen combination and glucose concentration were optimized for higher biomass production and results were pH 7, temperature of 37°C, 5 g/L glucose concentration and glucose and beef extract as glucose and nitrogen sources. PHA production in percentage was also obtained. The findings of the study indicated that PHA producing bacteria may be isolated from Boracads Beach in Batangas City which may be used as cheap substrate for PHA production.

Keywords: bioplastics, polyhydroxyalkanoates, Bacillus subtilis

INTRODUCTION

Plastics are synthetic products with basic materials derived from coal, natural gas and oil (Trivedi et al., 2016). These products are light weight, durable, cheap and are also useful and versatile (Ivar do Sul & Costa, 2013) materials since these are being used in everyday aspects of life and in the production of varying types

of products. Its demand in the market has been steadily increasing since their development 70 years ago (Jambeck et al., 2015). These are considered of great importance since their properties and performance are comparable to that of wood and metal (Arikan & Ozsoy, 2015), thus, its invention brought improvement to the quality of life of humans (Wong, Ngadi, Abdulla, & Inuwa, 2015). However, plastics are detrimental to the environment as the hefty use and production of plastics also increase waste (Sigler, 2014). Plastics are difficult to eliminate as their degradation time remains unknown (Li, Tse, & Fok, 2016), making them detrimental to the environment. Incineration or burning and burying which are common practices to reduce plastic waste contaminate both the soil and air (Almeida & Marques, 2016). Plastics can also be transported over long distances and may accumulate in bodies of water. Most of the plastic debris ends up in the marine environment that produces major impact on marine biota which may include inhalation, ingestion, smothering and may also modify the sea floor's composition (Gerber, 2015). Due to problems brought about by plastic use, development of bioplastics is gaining much interest (Ntaikou et al., 2014).

Bioplastics are substances that are biodegradable in nature (Trivedi et al., 2016), synthesized from biomass and renewable sources like oil, vegetable fats, corn starch and microbiota (Arikan & Ozsoy, 2015). Bioplastics take less time to break down which means less contribution to pollution and easier to recycle, making them environment friendly as compared to synthetic plastics (Reddy, Reddy, & Gupta, 2013). It also produce less carbon footprint, which results to lower energy cost in manufacturing and does not use scarce crude oil (Gill, 2014). Polyhydroxyalkanoates (PHA), polyactides, aliphatic polyesters, polysaccharides and copolymers are materials of bioplastics that are being studied for potential use (Mohapatra et al., 2015).

Polyhydroxyalkanoates or simply PHA are polyesters of hydroxyalkanoates that are synthesized by several gram positive and gram negative bacteria as storage materials for carbon and energy (Laycock, Halley, Pratt, Werker, & Lant, 2013). These are synthesized through a metabolic process with the use of different substrates like simple sugars, triacylglycerols and hydrocarbons (Jiang et al., 2016). These appear as intracellular granules that accumulate during times of depletion of essential nutrients like nitrogen, magnesium, phosphorus, and oxygen (Ong, Chee, & Sudesh, 2017) and during excess carbon sources (Kourmentza et al., 2017). PHAs may be classified as short-chain-lengths,

medium-chain-lengths and long-chain-lengths (Aslan, Ali, Morad, & Tamunaidu, 2016). Poly-3-hydroxybutyrate or PHB is the most common form of PHA (Chen, Hajnal, Wu, Lv, & Ye, 2015) and is the most studied member of the PHA family (Bugnicourt, Cinelli, Lazzeri, & Alvarez, 2014). These polyesters may accumulate to as much as 90% of the cells dry weight (Godbole, 2014). PHA consists of 100 different monomer units that create the possibility of creating varying types of biodegradable polymers (Singh, Kumar, Ray, & Kalia, 2015). It has numerous similar properties to that of materials used for the production of conventional plastics (Bhuwal, Singh, Aggarwal, Goyal, & Yadav, 2013). PHA being biodegradable, water-resisitant and oxygen permeable may also be applied in medicinal and pharmaceutical industry (Ntaikou et al., 2014). From brittle plastics to malleable and flexible elastomers, their properties are controlled by the type of substrate used, fermentation process and the microbe from which they originated (Dietrich, Dumont, Del Rio, & Orsat, 2015). These can be produced as environmental friendly as well as sustainable plastics through the process of fermentation (Wang, Yin, & Chen, 2014). Microbes that are capable of producing PHA are found in abundance in environments like estuaries, marine habitats, groundwater sediments and sewage (Koller, Marsalek, de Sousa, Miguel, & Braunegg, 2016).

Possible reservoirs of PHA-producing bacteria include environments rich in organic contents and nutrients that support the accumulation of PHA (Mohapatra et al., 2015). When cells do not get the nutrients necessary for normal metabolism, they undergo a state of stress (Tan et al., 2014). During this period they accumulate storage materials like pyrophosphate and PHA as a means of survival. The storage materials are produced when supply exceeds the immediate demands of the cell. PHA is stored in cells as granules to which the amount varies depending on the specie (Prados & Maicas, 2016). Nevertheless, PHA is a promising alternative to conventional plastics (Sabapathy, Devaraj, & Kathirvel, 2017) as it is an eco-friendly material that offers characteristics similar to that of plastics used nowadays (Cui, Zhang, Ji, & Wang, 2016).

The study aims to isolate PHA from bacterial strains grown in culture from samples derived from possible reservoirs of PHA-producing bacteria and to identify possible cheap substrates for PHA production. It also aims to identify strains of bacteria capable of producing PHA and to determine optimal conditions for PHA production in hope of future mass scale production. The study

would be limited to the isolation and biochemical characterization of PHA-producing bacteria as well as extraction and quantitative analysis of PHAs. This study would be of help in determining possible reservoirs of PHA producing bacteria in hope of finding an environmental friendly alternative to synthetic plastics.

MATERIALS AND METHOD
Sample Collection and Transportation (Mohapatra et al., 2015)

Water samples were collected from selected sites in Batangas City which include Boracads Beach in Tabangao and Tingga Falls in Tingga. Six water samples were obtained from Boracads Beach and were labeled as B1, B2, B3, B4, B5 and B6. On the other hand, four samples were collected from Tingga Falls and were labeled as T1, T2, T3 and T4. The samples were collected along the shores of the said places and placed in 50 ml sterile containers. The samples were transported to the laboratory for bacteriological analysis.

Isolation and Screening of PHA Producing Bacteria (Mohapatra et al., 2015)

Serial dilutions, 10^5 or 10^6, were prepared using 0.9% NaCl solution and 100 μl of diluted samples were spread on nutrient agar medium. Colonies of notable morphologies were individually picked and were subjected to PHA screening. Before screening, the isolates were induced to accumulate PHA granules in their cytosol using nitrogen limiting medium and incubated at 37 °C for 48 hours. The nitrogen limiting medium contained the following ingredients such as NaCl 3.0 g/L, KH_2PO_4 1.5 g/L, K_2HPO_4 1.5 g/L, $MgSO_4 \cdot 5H_2O$ 1.0 g/L, glucose 10.0 g/L, ammonium nitrate 0.5 g/L and agar agar 15.0 g/L. Smears were made using these bacterial isolates on a clean grease-free glass slide. After drying, 3% of Sudan black in 70% ethanol was added. The slide was immersed in xylene for few seconds followed with 5% Safranin for 20 seconds. It was then washed gently and observed under light microscope for detection of PHA granules in the cytosol of bacteria. All the Sudan Black B positive isolates were subjected to identification, extraction and quantification of PHA production.

Morphological and Biochemical Characterization (Mohapatra et al., 2015)

The morphological and physiological properties of PHA accumulating bacterial isolates were investigated on the basis of

their colony characteristics and gram's stain reactions using microscopic examination. The bacterial isolates were processed for identification by the standard methods of biochemical reactions using Simmons' Citrate Agar, Motility, Indole, Ornithine medium, Lysine Iron Agar, Triple Sugar Iron agar and Urea Agar.

Optimization of Growth Parameters for Biomass Production (Mohapatra et al., 2015)

Ecological deviation is one of the vital factors that determine the growth patterns of an organism. Thus, various parameters such as pH, temperature, sugar concentration, combination of different carbon and nitrogen sources were evaluated using marginally modified growth medium for biomass production. Moreover, some microbial strains have PHA accumulation which are parallel to biomass production. Thus, 24-hour fresh culture was cast-off to study the effect of parameters on PHA accumulating bacterial biomass production. The parameters include pH (6-10), temperature (23-51°C) glucose concentration (5-25 g/L), combination of different carbon (glucose, sucrose and fructose) and nitrogen sources (beef extract, peptone and yeast extract) in a constant ratio of 6:1.

To find out the optimum pH for biomass production, 100 ml of growth medium was taken in different test tubes and pH was adjusted from 6 to 10 by keeping glucose concentration at 5 g/L where glucose and yeast extract functioned as combination of carbon and nitrogen source respectively. One ml of 24-hour fresh culture was dispensed and incubated at 37°C for 24 hours. Then, comparative bacterial biomass production was evaluated. Similarly, other parameters were standardized following the mentioned methods.

Extraction and Quantitative Analysis of PHAs (Mohapatra et al., 2015: Rawte & Mavinkurve, 2002)

Owing to the above optimized growth parameters, bacterial isolates were grown in 1000 ml of modified growth medium for 72 hours at 37°C. Then, cell pellet was harvested by centrifugation at 6500 g rotation for I0 minutes and kept for drying. The dried weight of the pellet was taken as biomass weight. Subsequently, the cell pellet was re-suspended with sodium hypochlorite followed by incubation at 37°C for 10 minutes to digest the non-PHA materials. The mixture of sodium hypochlorite and bacterial biomass was centrifuged to harvest the PHA pellets which was then washed with cold diethyl ether. Finally, the pellet was centrifuged to

remove all the remaining organic solvents present with PHA pellets and was kept for air drying to yield dried form of extracted PHAs. PHA production was quantified using the following formula:

$$\text{PHA production (in\%)} = \frac{\text{Weight of PHA}}{\text{Weight of Biomass}} \times 100$$

RESULTS AND DISCUSSIONS
Sample Collection and Transportation
The six water samples obtained from the shore of Boracads Beach appeared as brownish in color, turbid with no noticeable odor. This may be due to the fact that Boracads beach is surrounded by houses and industrial factories like gas plantations which may have polluted the body of water. On the other hand, the four water samples obtained from Tingga Falls appeared as greenish brown in color, very turbid with no noticeable odor which may be caused by the surrounding houses and piggeries around the area.

Isolation and Screening of PHA Producing Bacteria
The four water samples collected from Tingga falls were all tested as negative in PHA screening while two out of six samples obtained from Boracads beach were tested as positive.

Table 1.Screening for PHA Production

| Sampling Site	PHA Screening
Tingga Falls	
T1	Negative
T2	Negative
T3	Negative
T4	Negative
Boracads Beach	
B1	Negative
B2	Negative
B3	Positive
B4	Positive
B5	Negative
B6	Negative

Legend: T=Tingga, B=Boracads

Table 1 shows the PHA screening results of the ten samples obtained from the sample sites. Among the ten water samples, samples B3 and B4 were screened as positive for PHA using the Sudan Black B staining method. PHA positive isolates showed black granules inside their cytosol as shown in Figure 1.

This means that PHA production is possible in the samples obtained from Boracads beach but is not possible in samples obtained from Tingga Falls. The positive PHA screening result of the samples obtained from Boracads beach is due to the fact that this body of water, being polluted, offers a limiting condition that favors the growth of PHA-producing bacteria. According to a study by Chaitanya, Nagamani, Rana, and Mahamood (2015), polluted bodies of water offers a kind of environment that has imbalanced nutritional contents that makes bacteria undergo stress and produce PHA as a coping mechanism.
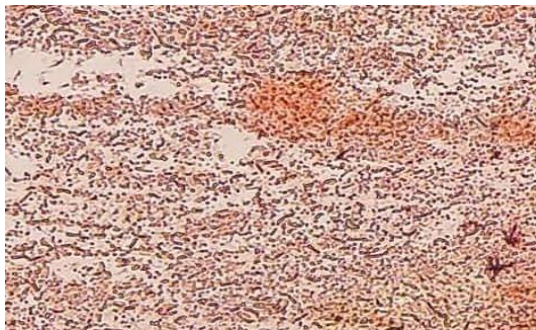
A. PHA granules of B3
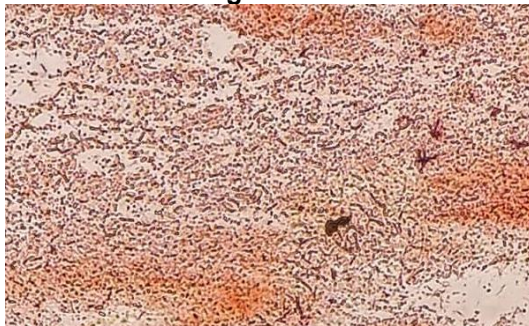
B. PHA granules of B4

Figure 1. PHA granules of samples B3 (A) and B4 (B)
Morphological and Biochemical Characterization
Water samples that tested positive in PHA screening were subjected to bacterial identification. The bacterium isolated from B3 was named as Isolate 1 while the bacterium isolated from B4 was named as Isolate 2. Table 2 shows the morphological and biochemical results of Isolates 1 and 2. Both isolates were gram-positive bacilli that tested positive for motility. Gas producton,

hydrogen sulfide production, pigment production, urease test and phenylalanine deaminase were all negative for both isolates. Glucose, sucrose and lactose fermentation were positive for both. The two isolates were identified as *Bacillus subtilis*. This isolate is proven to be a PHA producer as confirmed by past studies about PHA production (Singh, Patel, & Kalia, 2009). It is abundantly seen is soil and water and also inhabits extreme and contaminated environments. It is a nonfastidious bacteria capable of growing in simple materials. Since the samples were obtained from contaminated bodies of water which favors the growth of microbes capable of PHA production as proven by Mohapatra et al. in 2015.

Table 2. Morphological and Biochemical results

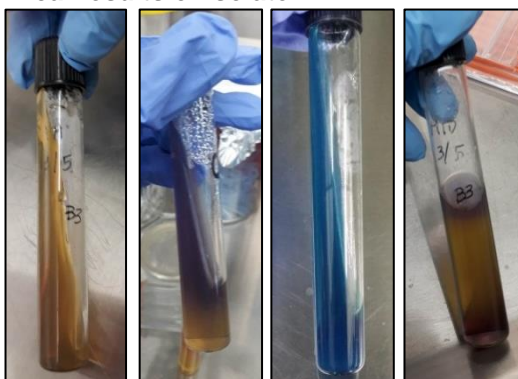|	**Isolate 1**	**Isolate 2**
Gram staining	Positive	Positive
Shape	Rods	Rods
Motility	Positive	Positive
Gas Production	Negative	Negative
Hydrogen Sulfide	Negative	Negative
Pigment	Negative	Negative
Urease	Negative	Negative
Glucose fermentation	Positive	Positive
Sucrose fermentation	Positive	Positive
Lactose fermentation	Positive	Positive
Phenylalanine Deaminase	Negative	Negative
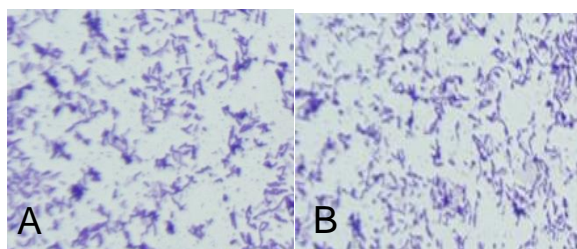
Figure 2. Gram Staining result of B3 (A) and B4 (B)
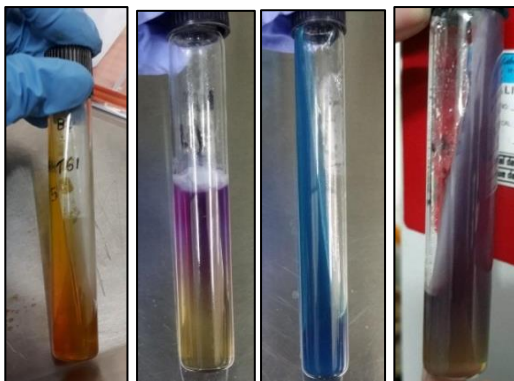
A. Biochemical results of Isolate 1

B. Biochemical results of Isolate 2

Figure 3. Biochemical results of Isolate 1 (A) and Isolate 2 (B)

Optimization of Growth Parameters for Biomass Production according to pH

The real challenge in PHA production is to be able to produce large amounts of PHA using cheap substrates for it to become useful. To be able to produce maximum amount of PHA, the isolates were grown in optimized culture media base on different parameters like varying pH (6−10), varying temperature (23°C−51°C), varying glucose concentration (5 g/L−25 g/L) and varying glucose (glucose, fructose and sucrose) and nitrogen sources (yeast, beef extract and peptone). *Bacillus subtilis* was used as the positive control to further confirm the identity of the isolates**.**
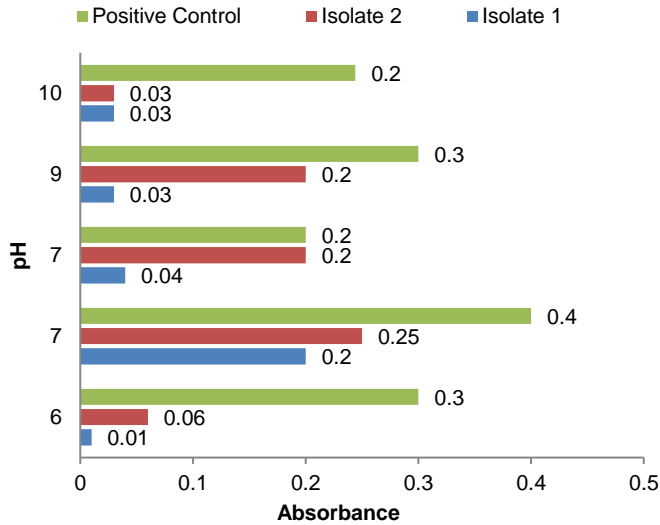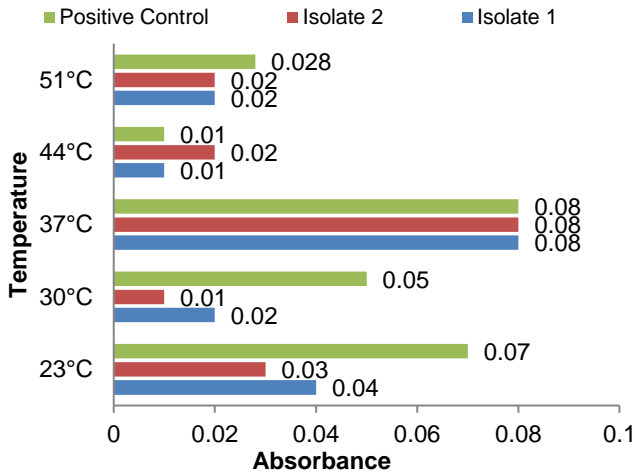
Figure 4. Optimization of Growth as to pH

Figure 4 shows that pH 7 has the maximum absorbance while pH 8 has the lowest absorbance. Absorbance is directly proportional to PHA production. This means that maximum PHA production was observed at pH 7 while the least amount of PHA was seen at pH 8. The results for pH agrees with the past studies about PHA production (Mohapatra et al., 2015), wherein pH 7 best supports the production of PHA. Furthermore, a study by Getachew and Woldesen (2016) revealed that PHA production achieves maximum amount at a pH range of 6.0-7.5. The same study also revealed that the variation of PHA production may be due to the fact that extreme pH may slow down metabolic activity of microorganisms resulting to lower production of PHA.

Optimization of Growth Parameters for Biomass Production according to Temperature

Another parameter for optimization of PHA production is temperature. Temperatures used were 23°C, 30°C, 37°C, 44°C and 51°C. Figure 5 shows that the temperature of 37°C has the highest absorbance while 44°C has the lowest absorbance.
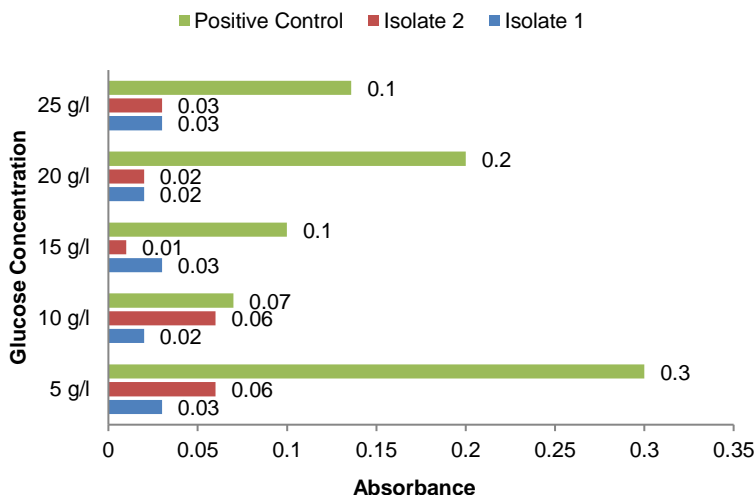
Figure 5. Optimization of Growth as to Temperature

This means that maximum PHA production was observed at the temperature of 37°C while the least amount of PHA production was observed at 44°C. production of PHA.The resulted temperature agrees with the past studies about PHA production (Mohapatra et al., 2015), wherein a temperature of 37°C best supports the production of PHA. Furthermore, a study by Getachew and Woldesen (2016) revealed that a temperature of 37°C best supports microbial growth and PHA production. The same study also revealed that the variation of PHA production may be due to the fact that extreme temperature may slow down metabolic activity of microorganisms resulting to lower

Optimization of Growth Parameters for Biomass Production according to Glucose Concentration

The third parameter tested is glucose concentration. Varying concentrations of glucose were used starting from 5 g/l up to 25 g/l. Figure 6 shows that 5 g/L glucose concentration recorded the highest absorbance while 10 g/L glucose concentration and recorded the lowest absorbance. This reveals that using 5 g/L glucose concentration produces maximum amount of PHA while 10 g/l glucose concentration produces the least amount of PHA.

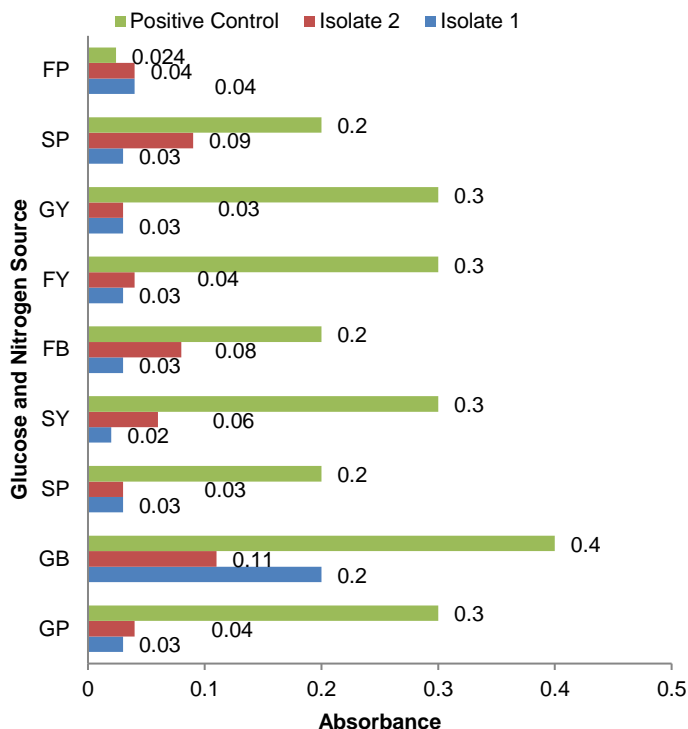**Figure 6. Optimization of Growth as to Glucose Concentration**

A study by Selim et al. in 2009 revealed that glucose concentration is directly proportional to PHA production until it reaches the maximum sugar it can consume. The same study also revealed that the optimum glucose concentration for PHA production is 5 g/l and PHA production started to decrease in subsequent concentrations when maximum amount of sugar was consumed.

Optimization of Growth Parameters for Biomass Production according to Glucose and Nitrogen sources

The last parameter tested was combination of glucose and nitrogen sources. The glucose sources used include glucose, fructose and sucrose while the nitrogen sources used include yeast, beef extract and peptone. Figure 7 shows that glucose and beef extract as glucose and nitrogen sources recorded the highest absorbance while fructose and peptone as glucose and nitrogen sources recorded the lowest absorbance.

Glucose is a monosaccharide and an easily assimilable source of carbon that encourages PHA production of bacteria resulting to a higher PHA yield (Getachew & Woldesen, 2016). On the other hand, sucrose being a disaccharide cannot be easily utilized by bacteria resulting to low PHA production (Elsayed, Aboshanab, Aboulwafa & Hassouna, 2013). Past studies showed

that beef extract, among other nitrogen sources also promotes the synthesis of PHA which explains as to why this combination of glucose and nitrogen source produced the highest amount of PHA as compared to other sources of nitrogen (Borah, Thakur, & Nigam, 2009).

Figure 7. Optimization of Growth as to Glucose and Nitrogen Sources

Extraction and Quantitative Analysis of PHAs

From the optimized parameters obtained, isolates were grown in modified media and PHA production in percentage was computed. Table 3 shows the PHA production of the isolates which were identified to be *Bacillus subtilis*. PHA production in percentage was computed and the isolates recorded PHA production of 40.86% and 40.75%, respectively while the positive control recorded a PHA production of 40.41%. A study by Singh, Patel and Kalia (2009) revealed that *Bacillus subtilis* has a percent PHA production ranging from 11-69%. The

positive control used was *Bacillus subtilis* which may further confirm that the isolates were the same with the positive control.

Table 3. PHA Production

	Biomass weight	Weight of PHA	% PHA Production
Isolate 1	0.042 g	0.0176 g	40.86 %
Isolate 2	0.034 g	0.014 g	40.75%
Positive control	0.021 g	0.0835 g	40.41 %

Statistical comparison of Isolate 1 and Isolate 2 versus the positive control were assessed if there is a significant difference in PHA production among the 3 isolates. Table 4 shows that there is no significant difference in PHA production both in Isolate 1 and Isolate 2 as compared with the positive control. This means that all 3 isolates produced the same amount of PHA which may further proved that all 3 isolates are *Bacillus subtilis.*

Table 4.Comparison of PHA Production (in %) across each Parameter

	P value	Interpretation
Isolate 1 vs Isolate 2	P > 0.05	Not Significant
Isolate 1 vs Positive Control	P > 0.05	Not Significant
Isolate 2 vs Positive control	P > 0.05	Not Significant

CONCLUSION

Polyhydroxyalkanoate or PHA has been extensively studied for its possible use as substitutes for production of plastics. The study revealed that Boracads Beach can be a source of substrate for PHA production. The resarchers were able to isolate *Bacillus subtilis*, which is considered PHA producer. Maximum PHA production was achieved using optimum conditions at pH 7, temperature of 37°C , glucose concentration of 5 g/dL and glucose and beef extract as glucose and nitrogen sources. Owing to the availability of such substrates, its utilization for PHA production would be of help in lowering the cost of PHA production.

RECOMMENDATION

The researchers recommended that more bodies of water around Batangas province be tested as potential substrates for PHA production. The experiment was limited only to samples

obtained near the shore, so the researchers recommended that comparisons be made from samples obtained from different depths and distances from the shore. Other samples like soil and wastewater may also be explored as possible sources of PHA producing bacteria. Furthermore, it is believed that the enzyme PHA synthase is the key enzyme in PHA production; thus, it is recommended that this enzyme be isolated on future studies about PHA.

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