SELECTION OF MEDIA, SUITABLE FOR *Azotobactervinelandii* BIOSYNTHESIS OF Poly-[3-hydroxybutyrate AND ITS EXTRACTION WITH CHLOROFORM.

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Abstract: Petroleum derived plastics constitute environmental problem because they are not biodegradable. Alternative substrates for manufacture of biodegradable plastics are necessary to safeguard the environment. Some bacterial genera produce Poly-p-hydroxybutyrate (PHB), under adverse nutrient conditions, which serve as alternatives for petroleum derived plastics. *Azotobacterspis* associated with its biosynthesis. Media suitable for growth of *Azotobactervinelandiiior* the accumulation of PHB are needed. In an effort to find out these facts, *Azotobacter* was isolated from rhizopheric soil of maize crop at Uli in Anambra State, Nigeria, by a selective media. Morphological characteristics were creamy beige colonies, Gram stain indicated negative rods. Biochemical tests were positive for catalase, oxidase, citrate, indole, sucrose, lactose, and hydrogen sulphide production, confirming the isolate to be *Azotobactervinelandiiby* Bergey's criteria. Growth of the isolate in acetate medium containing casitone as nitrogen source did not yield PHB. Combination of glucose and ammonium acetate medium containing castione gave the best yield of PHB per milliliter and per milligram of glucose consumed. Cell dry weight was 24.6% increase in GAAM and 22.50% in ACM. Treatment with sodium hypochlorite for 1 hour at 60°C lysed the cells of *A. vinelandii.* Addition of ethanol and acetone removed cell lipids and other molecules. Treatment with chloroform extracted PHB. Media combining glucose, ammonium acetate and casitone are suitable for *A. vinelandii* biosynthesis of PHB while chloroform extracts it.

Keywords: *Azotobactervinelandii,* chloroform extraction, poly-B-hydroxybutyrate biosynthesis, media selection.

INTRODUCTION:

Some bacterial genera have the ability to produce poly-beta-hydroxybutynate (PHB) when they are confronted with adverse nutrient conditions in their niches (Nandini*et al,* 2011; Wna*get al* 2009). These bacteria include *Azotobactersp,* pseudomonades (Chaitanya, *et al* 2014); *Ralstoriaeutropha, Alcaligeneseutrophus, Sinozhizobium*meliliti and recombinant *Escherichia coli* (Magada*et al,* 2013). PHB is the most common form of poly-hydzoxyalkanoates which are raw materials for the production of bioplastics. They arebiodegradable, biocompatible and thermoprocessibleplastic materials (Ueda

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et al; 1996, Hyakutate*et al*, 2011; Coaand Zhang, 2013). These qualities of PHB make it environmental friendly unlike the polypropylene derived from petroleum which is the opposite of PHB. There is increasing concern for the environment, to ensure that it does not degenerate. Therefore activities that encourage its sustainability are highly esteemed. The microorganisms that produce PHB can be grown in a variety of media, with different types of carbon sources. Magda *et al.*(2013) used a medium which was composed ofglucose (as the only carbon source), $MgSO_4$, NaCl2, $KH_2Po_4$and yeast extract to grow *Bacillus cereus*. Their pre-culture was nutrient broth. Gulab*et al.*(2013) used sugar industry waste with nutritive adjustment to grow *Bacillus subtilis*. Vaishnavi and Srividya (2013) used glocuse, $MgSO_4$, NaCl2, KH_2PO_4, peptone and yeast extract to grow *Bacillus thuringiensis*. Although it is more economical and environmental profitable to use waste organic sources, but in an initial study, it's safer to use substrates that have been documented with positive results before advancing to use newer ones. The extraction of PHB from the organisms can also be achieved by various methods.Vaishnavi and Srividya(2013), used sodium hypochlorite, incubated at 30°C, centrifuged at 10,000 rpm for 15min, water, and washed with acetone, methanol and diethyl ester. Magda *et al*, (2013), centrifuged at 4000rpm for 10min, incubated at 37°Cand used acetone, ethanol and water for the separation. In this work, the possible biosynthesis of PHB by *Azotobactervinelandii*grown in acetate medium containing casitone and another medium composed of glucose, ammonium acetate and casitone was investigated.

MATERIALS AND METHODS:

Sample Collection:

The soil sample from which the isolate was obtained was collected from rhizopheric soil of maize crop in a farm located withinChukwuemekaOdumegwuOjukwu University, Uli, Anambra State. Nigeria. A portion(1.0 g) of this soil sample was placed in a sterile testtube containing 9ml of sterile water. This formed the stock and from it, a 10-fold serial dilution was carried out up to 10^{-5} dilution.

Isolation of *Azotobacter*spp:

The medium used was nitrogen-free Jensen's agar medium according to Ninawe and Paurauj (1997). It wascomposed of sucrose 20g; KH_2PO_2 1g; $MgSO_4$, 0.5g; $NaCl_2$, 0.5g; $FeSO_4$0.1g; Na_2MoO_4 0.005g, agar, 20g, in 1l of distilled water. The pH was 7.3. After autoclaving at 121°C/15psi for 15min, it was allowed to cool to 47°C before pouring aseptically into sterile Petri dishes. This solidified Jensen's agar in Petri disheswere each subsequently inoculated with 0.1ml of 10^{-1}, 10^{-2} and 10^{-3} dilutions, and incubated at 30°Cfor 5 days. The isolate was further purified by several serial sub-culturing of the discrete colonies from the old culture on another fresh nitrogen-free Jensen's agar medium.

Identifications of isolates:

A modified method of Zohre*et al* (2012) was adopted for the identification. Morphological characteristics, biochemical tests and

sugar fermentation were the tools used in identifying the isolate. Their morphological characteristics included their colonial appearance on nitrogen-free Jensen's agar medium, and Gram reaction. The biochemical tests included *citrate, catalase, indole, oxidase* tests and test for H_2S production. The sugars used for sugar fermentation test were lactose and sucrose. For sugar fermentation, four test tubes were used. Each contained 10ml of Peptone water. To two of the test tubes were added 1.5g of lactose, 3 drops of 0.01% phenol red and the isolate. While to the remaining two tubes 1.5g of sucrose,three drops of 0.01% phenol red and the isolate were added. Inverted Durham tubes were also addedfor hydrogen sulphide production test; triple sugar iron agar slants prepared in tubes were streaked across thetop tubeof the slant and stabbed to the bottom of the tube with the isolate , and the tubes were incubated at 28°C for 24h.

Cultivation of *A. vinelandii* for PHB Biosynthesis:

The media used for the cultivation of *A. vinelandii*for the biosynthesis of PHB were glucose-ammonium acetate medium (GAAM) composed of 0.81mM $MgSO_4$, 0.58mM$CaSO_4$ in 5mM $KHPO_4$ buffer, pH was 7.2. The amount of glucose and ammonium acetate added were 3% (wt/vol.) and 15mM respectively. And acetate medium composed of 3% acetate, 0.81mM $MJgSO_4$, 0.58mM $CaSO_4$, 50mM ferric citrate 1mM Na_2MoO_4in 5mM $KHPO_4$ buffer, pH was 7.2. Bactocasitone (Difco certified), 0.1% (wt/vol, was added to the two media to promote PHB biosynthesis. These media were inoculated with 4% (vol. /vol.) of the isolate inoculums pre-grown for 48hrs in nitrogen-free Jensen's medium. These growth media (50 ml each) were placed in 250ml conical flasks and incubated at 25° to 30°C with slaking at 225rpm.

A. *vinelandii* growth Evaluation by Dry Weight Determination:

A. vinelandii growth in GAAM and ACM was monitored by measuring the spectrophotometric reading at 235nm of the culture at 0h of incubation and, that of the turbidity at the end of the incubation. The procedure involved centrifugation of the turbid culture at 225 rpm for 10min, discarding the supernatant and washing the cell pellet with distilled water. Then wet pellet was weighed to obtain the initial weight, before drying over night at 60°C until a constant weight was achieved.

Staining of Intracellular lipid:

Sudan black stain (Sudan black B powder 0.3g, 70% ethyl alcohol, 100ml) was used to stain the organism for the appearance of the intracellular lipid according to Magda *et al*, 2013.

Extraction of PHB:

A modified method of Magda *et al*, (2013) and Vaishnavi and Srividya(2013) were applied in the extraction. The bacterial cells were centrifuged for 45min at 6000rpm and were dried at 40° ± 1°Cfor 24h. The dried pellets were incubated at 60°Cfor 1h with sodium hypochlorite to lyse the cell wall of the organism. Supernatant was obtained by centrifugation at 6000rpm. Cell lipid and other molecules were extracted by adding 5ml of 96% (1:1 vol./vol.) ethanol and acetone. The PHB was extracted by hot chloroform

(adding 10ml chloroform in a water bath). The chloroform extract was dried at 40°C and 10ml of concentrated sulfuric acid was added. They were heated at 100°C in a water bath for 20min. After cooling to about 25°C, the mixtures were separated using separating funnel and the amount of PHB extracted was determined spectrophotometricallyat a wavelength of 235nm according to (Kurukoet *al*, 1989; Bowker, 1981; Ishizaki and Tanaka, 1991).

RESULTS:

Isolation:

The bacterial counts of each dilution of the isolate grown on nitrogen-free Jensen's medium are shown in table 1.

Table 1: Total Bacterial count of the samples

Days of Incubation	Dilution	Cfu/ml on NFM
5	10^{-1}	2.5×10^{3}
5	10^{-2}	1.6×10^{4}
5	10^{-3}	9.0×10^{4}

NFM = nitrogen-free medium

Identification of Isolates:

The results of the identification of isolates are presented in Table 2. The morphological characteristics showed creamy beige white colonies with raised elevation and smooth edge. The growth on NFM and the Gram stain is indicative of negative rods. The Biochemical test showed that the isolate was positive for catalase, oxidase, citrate, indole, sucrose, lactose and hydrogen sulphide production.

Table 2: Morphological and Biochemical Characteristics of Bacterial Isolates from Rhizospheric Soils of Maize Crop.

Morphological Characteristics						Biochemical Tests							
Isolate Code	Elevation	Pigmentation	Edge	Growth on NFM	Gram staining	Caltalase	Oxidase	Citrate	Indole	Succrose	Lactose	H_2S Product.	Probale gener
P1	Raised	Creamy beige white	Smooth	+	-ve rod	+	+	+	+	+	+	+	Azotobacter sp.
P2	Raised	Creamy beige white	Smooth	+	-ve rod	+	+	+	+	+	+	+	Azotobacter sp.

Cell dry Weight Determination Result.

The effect of the media on the growth of *A.vinelandii* as is reflected on the cell dry weight is presented on Table 4. Percentage increase in cell dry weight was higher in the culture grown on GAAM. In GAAM 24.6% increase was witnessed and in ACM, 22.50%.

Table 4: Cell dry weight of *A.vinelandii* due to effect of growth Media

Medium Type	Cell dry weight (g/l)	%Cell Dry Weight
GAAM	0.246*	24.60%
ACM	0.225*	22.50%

* This value is the mean of triplicate

Sudan Black stained *A. vinelandii Cells.*

The PHB granules stained blue black by Sudan Blackstain as viewed under oil immersion
(x 100). The cytoplasmic content stained light pink, **figure 1.**

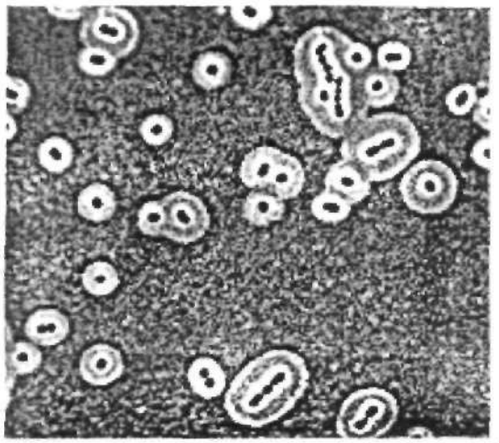

Figure 1: A micrograph of *A. vinelandii* stained with Sudan Black, showing the PHB granule in the center

The Yield of PHB at various Time Intervals The yield increased with increase in time. After 10 h of incubation, the yield was 0.05 mg/ml, while after 25 h it was 1.25 mg/ml, then after 45 h it was 1.92 mg/ml, figure2.

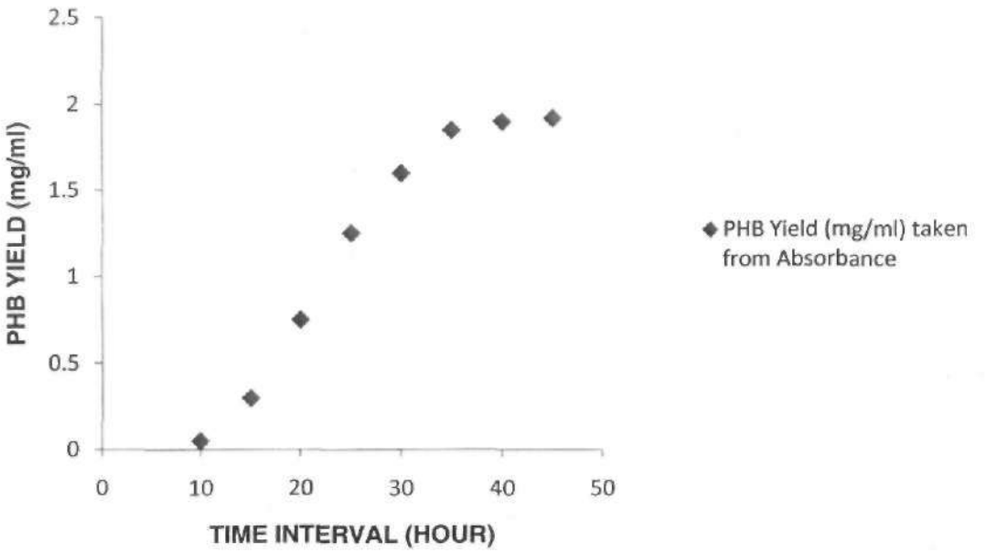

Figure 2: Amount of PHB biosynthesised at differnet time intervals in **GAAM.**

DISCUSSION

The medium used for the isolation of our organism was nitrogen-free Jensen's agar medium which is a selective

medium for the isolation of free living nitrogen fixing diautotrophs. *Azotabacters*pp belong to this group. The report showed that the counts ofthe isolate on this medium with respect to the dilution of the sample were 2.5 x 10^3Cfu/ml for 10^{-1} dilution, 1.6x10^4Cfu/ml for $10^{-2}$and 9.0 X 10^4Cfu/ml for 10^{-3} after 5days of incubation. (Table1). The colonial morphology of the isolates revealed only creamy beige white in all of the colonies and the edge/elevation were the same thoughtout. The Gram stain indicated the presence of Gram negative rod and the isolate tested positive for catalase, oxidase, citrate, indole, sucrose, lactose and hydrogen sulfide production reactions(Table2): confirming this isolate to be *A. vinelandii* according to Bergey's manual of determinative bacteriology. The presence of granular inclusions was also observed by Gram staining technique using Sudan black stain. The granular inclusions stained blue-black when viewed under oil immersion x 100 while the bacterial cytoplasm stained light pink; in agreement with the report of Nandini et al (2011). A graphical presentation of this image is presented on fig1. The *A. vinelandii* used in this work was pre-grown on nitrogen-free Jensen's agar medium. The only source of carbon was sucrose and the media used to grow *A. vinellandii*for PHB production were glucose ammonium acetate medium and acetate ammonium medium. The two media contained casitone. In the two media, culture turbidity increased significantly from 10h of incubation onwards and became milky white. In fig2 PHB content was highest when *A. vinelandii* was grown in glucose and ammonium acetate or casitone as nitrogen source. While growing in acetate medium with ammonium or casitone as the nitrogen source, PHB production was inhibited although there was evidence of cell growth (Table 4). The increase in percentage cell dry weight confirmed the support of GAAM for *A. vinelandii* growth. The PHB extracted from glucose ammonium acetate and read spectrophotometrically at 235nm was 0.229 mg/ml. showing that the extraction method adopted from Vaishnaviaid Srividya (2013) was adequate.

CONCLUSION:

These results show that *A. vinelandii* is capable of biosynthesizing PHB even in the presence of nitrogenous sources. Glucose was a better substrate, although the organism grew effectively in acetate medium. In conclusion,glucose can be exploited for the production of PHB.

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