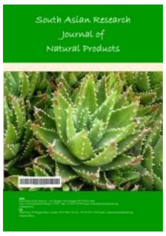

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Poly-3-hydroxybutyrate Biosynthesis by *Bacillus megaterium* Utilizing a Pleustophytic Ecological Plague in the Legendary Source of River Nile as the Sole Carbon Source

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Authors' contributions

This work was carried out in collaboration among all authors. Author TO designed the study. Authors TO and IM performed experimental and statistical analyses. Authors LNK, DMN, PN and AJ searched for relevant literature. Author TO wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Environmental isolates, genetically manipulated organisms, plants, animals and their products and economical methods are being expertly explored to biosynthesize poly-3-hydroxybutyrate plastics of comparable properties to petroplastics. This study assessed a hypothesized feasibility of utilizing water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laubach) from Lake Victoria (Uganda) as a potential carbon source for poly-3-hydroxybutyrate biosynthesis. The poly-3-hydroxybutyrate

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biosynthesizing bacteria (*Bacillus megaterium*) was isolated from municipal sewage sludge and harnessed for batch fermentation of acid-catalysed water hyacinth biomass. Poly-3-hydroxybutyrate formed in the cytoplasm of the bacterial cells was extracted by chloroform extraction method, and thereof confirmed and quantified by UV spectroscopy. Batch fermentation was carried out in 100 ml of the culture media for different times (48, 96, 144 and 192 h) to determine the best incubation time for maximum yield. A maximum yield of 61.3% was realized after 96 h of fermentation beyond which the bioplastic yield started decreasing. Utilization of this ecological plague for poly-3-hydroxybutyrate biosynthesis is a promising strategy for regulating the weed population along the length of River Nile and the Victorian basin.

Keywords: Batch fermentation; Lake Victoria; poly-3-hydroxybutyrate; sewage sludge.

1. INTRODUCTION

Water hyacinth is an invasive greenery with credited floral beauty on Lake Victoria. It is a perennial aquatic herb of the pickerelweed family (Pontederiaceae) that is native to tropical America [1]. It is reported to have been introduced into East Africa as an ornamental plant and it spread into Lake Victoria through the Kagera river [1]. The weed has flourished due to the suitable growth conditions, total absence of omnivorous predators and heavy metal pollution of Lake Victoria.

In 1995, the weed choked 90% of the Victorian shoreline with giant mats reported in hectarages of Murchison, Wazimenya and Gobero bays. Port Bell (Luzira) and Kasensero (Rakai) of Uganda are among the frequently hit landing sites by the weed resurgence [2]. In Uganda, resurgence was still observed on Kagera river, MacDonald, Fielding, Bunjako, Murchison, Lwera, Napoleon Gulf, Berkeley and some Ssesse Island bays in 2012 [3]. The weed has since threatened the harvest of tilapia (*Oreochromis niloticus*), Nile perch (*Lates niloticus*) and silver fish (*Rastreneobola argentea*) [4]. Water hyacinth is a menace, impedes boat access, block communal water points along Victorian shorelines [2] and in prolific cases increase the spread of diseases such as bilharzia, malaria [5], skin rush, cough, encephalitis and digestive disorders. It also hamper water treatment, hydroelectricity generation and irrigation operations [6]. Floating mats of the weed curtail light penetration into Lake Victoria, limiting growth of photosynthetic phytoplankton [1].

Although it is a deleterious aquatic weed, water hyacinth is a good source of biogas [7] and has been utilized in phytoremediation of iron, zinc, sodium, potassium, magnesium, calcium, lead, mercury and strontium-90 in matrices [8-12]. Water hyacinth is a substrate for the production of various furniture, handbags, ropes, potash, livestock feed [13], biofertilizers [14,15], papers [16], superabsorbent polymers and poly-3-hydroxybutyrate plastics [17-19].

Poly-3-hydroxybutyrate (PHB) is a fully decomposable member of the biopolyester family with optical activity, piezoelectricity and excellent barrier properties. It is a partly crystalline thermoplastic with properties comparable to those of isotactic polypropylene and other elastomer petroleum-based plastics. In addition, PHB has low permeability for water, molecular oxygen and carbon dioxide [20]. PHBs are produced by a range of microbes cultured under different nutrient and environmental conditions [21]. The biopolyesters are harnessed as energy storage materials by microbes in transient abundance of carbon with nutritional components such as phosphorous, magnesium, oxygen, nitrogen or sulfur being a limiting factor [22]. They accumulate intercellularly as liquid, mobile or amorphous granules and may be deposited in an amount equivalent to 90% of cellular dry weight [23]. Poly-3-hydroxybutyrates are biodegraded within 5-6 weeks releasing carbon dioxide and water [24]. Their biocompatibility coupled with low oxygen permeability makes them suitable for medical applications as biodegradable carriers, surgical needles, surgical suture materials, bone tissue substitutes, osteosynthetic materials, bone plates, rivets and tacks [25].

Several studies have reported on PHB biosynthesis using various carbon sources. In a study, the accumulation of PHB granules in the cells of *B. megaterium* ATCC 6748 was reported to entirely rely on the ratio of carbon and nitrogen sources [26]. The investigation utilized sugarcane molasses (MOL) and corn steep liquor (CSL) as renewable sources of carbon and nitrogen respectively. The highest PHB yield (43% w/w, dry matter) was observed after 45 hours of microbial growth when equal quantities (4%) of MOL and CSL were experimented whereas the highest biomass (7.2 gL^{-1}) was recorded at 4% MOL and 6% CSL. The team concluded that bacterial growth increased as CSL concentration increased and PHB accumulation contrarily decreased. The chemical structure and thermal properties of PHB produced were comparable to that of the commercial PHB except for the significantly higher molecular mass and lower degree of crystallinity.

The effect of various carbonaceous and nitrogenous sources on PHB production was investigated by Gouda, et al. [27]. The highest yields of 40.8% and 39.9% per mg cell dry matter were achieved with cane molasses and glucose respectively. Optimum growth was achieved with 3% molasses with maximum yield of 46.2% per mg cell dry matter of PHB. Corn steep liquor was the most sustainable synthetic nitrogen source with a yield of 32.7% per mg cell dry matter. Optimal growth was achieved with chloride, sulphate, oxalate or phosphate of ammonium ion used as the chief nitrogen source.

A *novel B. megaterium* strain was isolated and characterized by López et al. [28]. Its ability to biosynthesize PHB was assessed using various fermentation configurations on formulated media. The novel strain gave 59% and 60% PHB yield of its dry cell weight in bioreactor assessments utilizing glucose and glycerol as the chief carbon sources. Basing on ^{13}C NMR and FTIR analyses, they concluded that despite the fact that the novel strain sporulates, its intracellular PHB biosynthesis potential was higher than those previously reported in literature. Another study conducted by Rodrıguez-Contreras, et al. [29] with a novel *B. megaterium* strain (uyuni S29) for PHB biosynthesizing capacity reported a future of considering the strain for industrial PHB production. The strain gave 70% yield in a fermentation reactor against 60% of biosynthesized polymer that is necessary for recommending a strain as economical for large scale biosynthesis of PHB [30,31]. More so, the industrial conditions utilize conventional medium and moderate salt content, an environment that was already replicated in their previous study [29] and carried on in the aforeacknowledged study.

Unfortunately, PHB production is not economical due to its prohibitive production cost. The current efforts of researchers aim at reducing the cost of production through identification of efficient bacterial strains [20,32] and cheap substrates. This study reported the feasibility of using water hyacinth for batch biosynthesis of PHB using *B. megaterium* isolated from municipal sewage sludge.

2. MATERIALS AND METHODS

2.1 Sampling of Water Hyacinth and Sewage Sludge

Water hyacinth (5 kg) was collected from Port Bell, Luzira, Kampala, Uganda where one of the recent resurgences was reported [2]. It was washed several times with distilled water, oven dried at 70ºC for 48 h and ground into fine powder. Sewage sludge (5 kg) was collected from Lubigi Sewage and Faecal Sludge Treatment Plant, Kampala, Uganda [33] in sterile paper bags and microbiologically analyzed within 2 hours of collection.

2.2 Isolation of PHB Biosynthesizing Bacteria and Preparation of Carbon Source

Poly-3-hydroxybutyrate biosynthesizing bacteria were isolated from sewage sludge, purified and identified as *B. megaterium* following standard methods [34]. The purified isolates were cross streaked on nutrient broth (2.5 g/L peptone, 2.5 g/L NaCl, 1.0 g/L yeast extract and 0.5 g/L beef extract). Measured 100 ml of the culture in a 250 ml Erlenmeyer flask was inoculated with a 2% v/v inoculum and incubated at 37ºC for 12 h with orbital shaking at 230 rpm.

Substrate hydrolysate preparation was performed following a modified analytical procedure advanced by Pumiput, et al. [35]. Aliquots (8.0±0.1 g) of powdered water hyacinth leaves were steam exploded in an autoclave at 121ºC for 20 min. Distilled water was added to the wet pretreated powder in a 250 ml volumetric flask to top up the volume to the mark. The resultant mixture was subsequently boiled at 80°C for 30 min and the hydrolysate recovered by filtration. Acid post-hydrolysis of the hydrolysate was performed to split the oligosaccharides in the hydrolysate to monomeric sugars by autoclaving at 121ºC with 1% hydrochloric acid (v/v) for 30 min. The pH of the resultant hydrolysate was adjusted with sodium hydroxide to 7.0 and the precipitate recovered by filtration [18,35].

2.3 PHB Production, Extraction, Purification and Quantification

Preliminary screening for the detection of bacterial isolates capable of biosynthesizing and accumulating PHB was performed following the analytical procedure used by Zhang, et al. [36]. Batch fermentation was done in a 250 ml Erlenmeyer flask containing 100 ml of nitrogen-deficient culture media. The flask was inoculated and maintained at 30ºC with orbital shaking at 130 rpm for 48, 96, 144 and 192 h.

For analysis, the samples were centrifuged for 45 min at 6,000 rpm. Obtained pellets were incubated at 60ºC for 1 h with sodium hypochlorite to break the cell walls of bacteria. Supernatants obtained were transferred to a Soxhlet system. Cell lipids and other molecules (except PHB) were extracted by addition of 5 mL of 96% ethanol and acetone (1:1 v/v). PHB was extracted using chloroform, dried at 40ºC followed by addition of 10 mL of concentrated sulfuric acid. The resultant solution was heated in a water bath at 100ºC for 20 min.

After cooling, quantification of biosynthesized PHB was performed employing an analytical procedure used in previous studies [18,37,38]. The biopolymer was quantified using a double beam optimal geometry Genesys 10S UV visible spectrophotometer (Thermo Scientific, USA) in comparison with a standard curve plotted between concentrations of crotonic acid and the corresponding absorbances were read at 235 nm. For dry cell weight (DCW) analysis, 10 mL of culture sample was centrifuged at 11,200 × g for 20 min. The cell pellet was washed twice with 1 mL of distilled water and transferred to a dry petri dish. The pellet was dried to constant weight at 60ºC to estimate the DCW in g/mL. Three independent replications were performed and the percentage of PHB accumulated was estimated using Equation 1.

$$\text{PHB accretion} = \frac{\textbf{Dry weight of PHB}}{\textbf{Dry cell weight}} \times 100\% \qquad (1)$$

2.4 Characterization and Confirmation of the Extracted Polymer

Characterization and confirmation of PHB recovered was done using crotonic acid assay. The powder was dissolved in sulphuric acid (1 mg/mL) and heated at 100ºC for 10 min to convert it into crotonic acid. The solution was cooled, and its spectroscopic absorbance read at 260 nm against concentrated sulphuric acid as blank.

2.5 Analytical Quality Control and Quality Assurance

All reagents used were of analytical grade. The volumetric ware used were soaked overnight in 10% (v/v) nitric acid solution, rinsed with deionized water and oven dried prior to analysis. A calibrated Mettler PM200 digital analytical balance (Marshall Scientific, Hampton, USA) was used for all weighings. Hanna 211 digital microprocessor-based bench top pH/mV/ ℃ meter (Hanna instruments, Italy) calibrated using pH 4.01, 7.01, 10 buffers was used for all pH measurements.

2.6 Statistical Analysis of Results

Results from triplicate analyses were subjected to statistical evaluation. One-way ANOVA was done followed by Turkey pairwise test to separate the means. All statistical analyses were performed at a 95% confidence interval in the general linear model using Sigma Plot statistical software (v14.0, Systat Software Inc., San Jose, CA, USA) [39].

3. RESULTS AND DISCUSSION

3.1 Bacterial Staining and Hydrolysis of Water Hyacinth

The use of the lipophilic stain (Sudan black) to stain intracellular polyhydroxybutyrate (PHA) granules accumulated by the isolated *B. megaterium* was a confirmatory test that the isolated bacteria was a PHA biosynthesizing species [40]. The bacterial colonies were bluish black and the PHB granules were confirmed by their affinity for Sudan black dye [41]. The acid hydrolysis method of Pumiput, et al. [35] which was used while investigating lactic acid production from fruit waste registered success in hydrolyzing water hyacinth biomass in this study. This agreed well with the study of Preethi et al. [18].

3.2 Recovered Poly-3-hydroxybutyrate and its Accretion

Use of organic solvents such as chloroform for PHB extraction is one of the most employed analytical procedures for recovering PHA and thus PHB. It is published that chloroform alters cell membrane permeability of the PHA biosynthesizing bacterial cells and subsequently solubilize the PHA component, releasing it in solution [42]. The average yields of PHB from the cells are presented in Table 1.

Table 1. Poly-3-hydroxybutyrate yield of the water hyacinth hydrolysate

Incubation time (hours)	Recovered PHB (g/L)*	PHB accretion (%)
48	1.2 ± 0.06	15.0
96	4.9 ± 0.12	61.3
144	4.2 ± 0.06	51.3
192	3.8 ± 0.12	47.5

**Presented as mean ± standard error of triplicates*

There was gradual increase in PHB biosynthesis by *B. megaterium* in the water hyacinth medium. A high yield of PHB (4.9±0.12 g/L, 61.3%) was realized on the fourth day of fermentation (96 hours) in water hyacinth medium. The result of this investigation is corroborant with that observed with *Cupriavidus necator* [43] and *Pseudomonas aeruginosa* [18] where PHA yield was 4.3 g/L. Yüksekdağ, et al. [35] reported that *B. megaterium* 12 produced 0.142 g/L of PHB with a yield of 14.79% after 45 hours with a significant reduction in PHB yield after 48 hours. Increase in the fermentation time in this study resulted in a significant decline (*P = .05*) in PHB biosynthesis. This could be correlated with the utilization of intracellular PHA granules as reserve food molecules during nutrient starvation [44]. Thus, it can be thought that until sporulation time, the bacteria produced PHB and subsequently used it**.** The decrease in polymer yield after the 96th hour is indicative that the biosynthesizing bacteria utilized the polymer as a source of carbon and nitrogen, triggering unfavorable growth conditions due to depletion of carbon and nitrogen sources in the hydrolysate medium. It is reported that bacterial spores are produced during the stationary phase as PHB is being biosynthesized and utilized [45,46]. The results of this study is comparable to that of Klüttermann, et al. [47] who reported that *Agrobacterium radiobacter* gave a maximum accretion of 60% PHB of cell dry weight in the stationary growth phase after 96 hours with a significant drop in yields reported after this time. Reddy, et al. [48] also reported that *B. megaterium* strain OU303A from sewage sludge successfully biosynthesized PHB and polyhydroxybutyrate-co-hydroxyvalerate (PHB-co-HV) copolymer. The strain had an all-out yield of 62.43% DCW polymer in a medium containing glycerol as the sole carbon source, comparatively higher than 58.63% DCW polymer in glucose as the sole carbon source. Additionally, the strain reportedly produced 2.5% hydroxyvalerate copolymer from glucose with increase in hydroxyvalerate monomer yield following the inclusion of its copolymer precursor in the fermentation medium.

Polyhydroxyalkanoates can be chemically converted to crotonic acid by heating in concentrated sulphuric acid. The UV spectroscopic absorption maximum of crotonic acid is normally shifted to 260 nm when concentrated sulphuric acid is used as the solvent [40]. Carboxyl compounds absorbs light below the UV range and hence are difficult to detect by spectroscopy. Crotonic acid assay relies on the chemical fact that UV absorption maxima of alpha and beta unsaturated acids undergoes a strong bathochromic shift (shifts to lower frequency) in sulphuric acid and can be recorded in the UV range; the corresponding absorption maximum is thus shifted to 260 nm [40]. This study confirmed that PHA, a PHB precursor was formed from fermented water hyacinth which is corroborant with preceding studies [18,49-51].

4. CONCLUSION AND RECOMMENDATION

From this study, it was evidential that water hyacinth is a potential candidate for batch production of PHB and the yield of PHB increased with increase in fermentation times. A maximum yield of 61.3% per dry cell mass was obtained after 96 hours of fermentation. Increase in fermentation time beyond 96 hours did not register any increment in PHB yield. The use of water hyacinth as a starting substrate for PHB biosynthesis using *B. megaterium* isolated from sewage could be a feasible strategy for managing the population of the noxious weed in the Victorian basin and the entire River Nile length. Further research should identify the strain of *B. megaterium* harnessed from the sewage sludge as well as determine the nutritive parameters of the water hyacinth leaves.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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