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Poly-3-hydroxybutyrate Biosynthesis by Municipal Sewage Sludge Isolated *Bacillus megaterium* Utilizing a Pleustophytic Ecological Plague in the Legendry Source of River Nile as the Sole Carbon Source

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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 a proliferative pleustophytic greenery, water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laubach) in Lake Victoria, Uganda as a potential carbon source for poly-3-hydroxybutyrate biosynthesis. The poly-3-hydroxybutyrate 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 100ml of the culture media in a 250ml fermenter for different times (48, 96, 144 and 192 hours) to determine the best incubation time for maximum yield. An all-out net yield of 61.3% was realized after 96 hours of fermentation. Utilization of this ecological plague for poly-3-hydroxybutyrate biosynthesis is a promising strategy for regulating the weed population along the length of Nile River and the Victorian basin.

Keywords: *batch fermentation, Lake Victoria, ornamental verdure, poly-3-hydroxybutyrate, sewage sludge*

1.0 Introduction

Water hyacinth is a pernicious invasive and cosmopolitan greenery with credited floral beauty on Lake Victoria. It is a perennial aquatic herb of the pickerelweed family (Pontederiaceae) and is a native of tropical America [1]. It is attested to have been introduced into East Africa as an ornamental verdure and progressed into the world`s second largest fresh water lake via the Rwandese Kagera river [1]. Owing to the available suitable growth

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conditions, total absence of omnivorous predators, elevated trace metal pollution and spacious environment of Lake Victoria, the plant has flourished [1]. The plant itself is morphologically plastic, propagates prolifically and is highly flexible to variations in temperature, light intensity, water pH and salinity [2]. Thus, it has a swift mode of proliferation, enabling it to adapt to distant dispersal and colonization of various aquatic niches.

In 1995, the weed choked 90% of the Victorian shoreline with giant mats witnessed 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 [3]. In Uganda, resurgence was still observed on Kagera River, MacDonald, Fielding, Bunjako, Murchison, Lwera, Napoleon Gulf, Berkeley and some Ssesse Island bays in 2012 [4] and has since threatened the harvest of tilapia, Nile perch and silver fish (omena) [5].

The weed is a menace, impedes boat access, block communal water points along Victorian shorelines [3] and in prolific cases increase the rate of spread of diseases such as bilharzia, malaria [6], skin rush, cough, encephalitis and digestive disorders. It also hampers water treatment, hydroelectricity generation and irrigation operations [7]. Floating mats of the weed curtail light penetration into L. Victoria, limiting growth of photosynthetic phytoplankton [1].

Although it is a deleterious aquatic weed, it is an excellent source of biomass with an estimated hectare (ha) yield of $7x10^4 m^3$/ha of biogas composed of 70% methane and 30% carbon dioxide [8]. According to Curtis and Duke [9], a kilogram of water hyacinth dry matter yields 370 litres of biogas, with a heating value of 22,000 kJ/m^3, far better than pure methane gas. *E. crassipes* is reported to bioremediate trace metals: iron, zinc, sodium, potassium, magnesium and calcium in distillery effluent [10] and Lead, Mercury, Strontium-90, carcinogenic organic compounds, nitrogen [11-13] and potassium [14] in water. The weed leaf concoction is phytotoxically effective against *Mimosa pigra* and *Vigna radiata* [15]. Water hyacinth is a starting material for the production of various furniture, handbags, ropes, potash, livestock feed [16] for pigs (though it is reportedly not so palatable owing to the presence of oxalate crystals in the leaves that cause mouth irritation), biofertilizers (compost or mulch) [17-19], paper manufacture [20], superabsorbent polymer material synthesis [21] and biosynthesis of poly-3-hydroxybutyrate bioplastics due to its high carbonaceous content [2,22].

1.1. Poly-3-hydroxybutyrate

Poly-3-hydroxybutyrate (frequently called poly-beta-hydroxybutyrate) is a fully decomposable member of the biopolyester family with optical activity, piezoelectricity and excellent barrier properties. It is a partly crystalline thermoplastic with a high melting point and its biosynthesis is sustainable for voluminous production at economically reduced costs [23]. Poly-3-hydroxybutyrate, the simplest and most encountered polyhydroxyalkanoate (PHA) have **x** and **R** in the general structure of PHAs, $-[O\text{-}CH(\mathbf{R})(CH_2)_xCO]-$ equal to 1 and CH_3 respectively [24]. Its properties are comparable to those of isotactic polypropylene and other elastomer petroleum-based plastics thus is gaining attention as a substitute for these plastics [24-26]. In addition, poly-3-hydroxybutyrate has low permeability for water, molecular oxygen and carbon dioxide.

Polyhydroxyalkanoates are produced by a range of microbes, cultured under different nutrient and environmental conditions [27]. The biopolyesters are harnessed as energy and carbon storage materials by the microbes in transient abundance (excess) of carbon source with nutritional components such as phosphorous, magnesium, oxygen, nitrogen or sulfur being a limiting factor [28-34]. The biopolymers, naturally lipids, accumulate intercellularly as liquid, mobile or amorphous granules to ensure survival under physiological stress by the

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microbe and may be deposited in an amount equivalent to 90% of cellular dry weight [35]. Poly-3-hydroxybutyrates have melting points between 40-180°C; are biodegraded in microbe active environments within 5-6 weeks releasing carbon dioxide and water [36,37]. Their biocompatibility coupled with low oxygen permeability makes them suitable for medical applications thus can be used as implants without inflammatory side effects. Other bioplastic applications of poly-3-hydroxybutyrate include its utilization as biodegradable carriers, surgical needles, surgical suture materials, bone tissue substitutes, osteosynthetic materials, bone plates, rivets and tacks [38].

1.2. Recent Studies Done on Poly-3-hydroxybutyrate Production

Yüksekdağ and others [39] experimented poly-3-hydroxybutyrate production by two *Bacillus* species *(subtilis* 25 and *megaterium* 12) in nutrient broth at various times between 6 and 48 hours. Their findings revealed 1.01×10^{-1}g/L, 1.42×10^{-1}g/L production of poly-3-hydroxybutyrate with 18.03% and 14.79% yields after 45 hours respectively. After 48 hours, a significant reduction in poly-3-hydroxybutyrate yield was observed. Whereas Poly-3-hydroxybutyrate accumulation in the culture broth by the strains were nearly insignificant with the two carbon and nitrogen sources, the highest poly-3-hydroxybutyrate level was noted in protease peptone enriched medium. In this enriched broth, poly-3-hydroxybutyrate yield of *B. subtilis* 25 was 78.69%, whereas in the same nitrogen sources, *B. megaterium* 12 had 77% yield [39].

The accumulation of poly-3-hydroxybutyrate granules in the cells of *B. megaterium* ATCC 6748 was reported to entirely rely on the ratio of carbon and nitrogen sources by Chaijamrus and Udpuay [40]. The investigation utilized sugarcane molasses (MOL) and corn steep liquor (CSL) respectively as renewable sources of carbon and nitrogen. The highest poly-3-hydroxybutyrate production (43% w/w, dry matter) was observed after 45hours of microbial growth when equal quantities (4%) of MOL and CSL were experimented, whereas the highest biomass ($7.2gL^{-1}$) was recorded at 4% MOL and 6% CSL. The team concluded that bacterial growth increased as CSL concentration increased and poly-3-hydroxybutyrate accumulation contrarily decreased. The formation rate of poly-3-hydroxybutyrate up to 0.016 hr^{-1} and specific growth rate of up to 0.25 h^{-1} were reported during the experimental growth [40]. The chemical structure and thermal properties of poly-3-hydroxybutyrate produced from MOL and CSL were comparable to that of the commercial poly-3-hydroxybutyrate, except for the significantly higher molecular mass (about 3.9 x 10^6 Da) and lower degree of crystallinity.

The effect of various carbonaceous and nitrogenous sources on poly-3-hydroxybutyrate production was investigated by Gouda *et al* [41]. The highest production of 40.8% and 39.9% per mg cell dry matter was 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 poly-3-hydroxybutyrate was achieved with 2% molasses. Corn steep liquor was the most sustainable synthetic nitrogen source with a yield of 32.7 mg per cell dry matter. Optimal growth was achieved with the chloride, sulphate, oxalate and/or phosphate of Ammonium ion used as the chief nitrogen source.

A novel *B. megaterium* strain was isolated and characterized by López *et al* [42]. Its probable ability to be utilized in poly-3-hydroxybutyrate production was assessed using various fermentation configurations on formulated media. The novel strain gave 59% and 60% poly-3-hydroxybutyrate yield of its dry cell weight in bioreactor assessments utilizing glucose and glycerol as the chief carbon sources. Basing on carbon-13 Nuclear Magnetic Resonance and Fourier Transform Infrared analyses, they concluded that despite the sporulation

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phenomenon exhibited by the novel *Bacillus* strain, its intracellular Poly-3-Hydroxybutyrate biosynthesis potential was higher than those previously reported in literature.

A study conducted by Rodrıguez-Contreras *et al* [43] with a novel *B. megaterium* strain (uyuni S29) for poly-3-hydroxybutyrate biosynthesizing capacity reported a future of considering the strain for industrial poly-3-hydroxybutyrate production. The strain gave 70% Poly-3-Hydroxybutyrate yield in a fermentation reactor against 60% of biosynthesized polymer that is necessary for recommending a strain as economical for large scale biosynthesis [44,45]. More so, the industrial scale conditions utilize conventional medium and moderate salt content; an environment that was already replicated in their previous study [46] and carried on in the aforeacknowledged study.

Unfortunately, voluminous production of poly-3-hydroxybutyrate is not industrially economical due to its prohibitive production cost. The current efforts of researchers aims at reducing the cost of production through identification of efficient bacterial strains [26,46] and potentially cheap substrates. The genus *Bacillus* received attention [47] due to the stability of its replication and plasmids maintenance. Many *Bacillus* species have reported poly-3-hydroxybutyrate production potential [48,49]. This study reported the feasibility of utilizing the pleustophytic ecological plague, *Eichhornia crassipes* (Mart.) Solms-Laubach in Lake Victoria, Uganda for batch biosynthesis of poly-3-hydroxybutyrate using *B. megaterium* isolated from municipal sewage sludge.

2. Method

2.1. Isolation of Poly-3-Hydroxybutyrate Biosynthesizing Bacteria

Sewage sludge samples were collected from Lubigi Sewage and Faecal Sludge Treatment Plant (SFSTP). The treatment plant, operated by National Water and Sewerage Corporation (NWSC), Kampala, Uganda receives and treats wastewater from a piped network as well as faecal sludge that is brought by private cesspool emptiers. The plant provide 400m^3 per day capacity for faecal sludge treatment [50].

The sludge samples were collected in sterile paper bags and microbiologically analyzed within 2 hours of collection. The bacteria was isolated and identified as *B. megaterium*. Bacterial isolates from the sludge was cross-streaked on nutrient broth (2.5g/L peptone, 2.5g/L NaCl, 1.0g/L yeast extract and 0.5g/L beef extract). 100ml of the cultures in a 250ml Erlenmeyer flask was inoculated with a 2% v/v inoculum and incubated at 37°C for 12 hours with vigorous orbital shaking at 230 rpm.

2.2. Collection and Preparation of the Carbon Source

Water hyacinth was collected from Port Bell, Luzira, Kampala-Uganda where one of the recent resurgences have been reported [3]. It was washed several times with distilled water and oven dried at 70°C for 48 hours. Fine powdered water hyacinth was utilized as the sole carbon source.

Substrate hydrolysate preparation was performed following a modified analytical procedure of Pumiput *et al* [51]. Aliquots (8.0±0.1g) of a fortnight shade dried powder of water hyacinth leaves (Figure 1 (a)) was steam exploded in an autoclave at 121°C for 20 minutes. Distilled water was added to the wet pretreated powder in a 250ml volumetric flask to top up the volume to the mark. The resultant mixture was subsequently boiled at 80°C for 30 minutes and the hydrolysate was recovered by filtration.

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Acid post-hydrolysis of the hydrolysate was performed to split the oligosaccharides in the hydrolysate to monomeric sugars by autoclaving at 121°C with 1% hydrochloric acid (v/v) for 30minutes. The pH of the resultant hydrolysate was adjusted with Sodium hydroxide to 7.0 and the precipitate recovered by filtration through Whatmann No.1 filter paper (Figure 1 (b)) [2,51].

(a) **(b)**

Figure 1. Water Hyacinth (a) Powdered samples (b) Hydrolysates

2.3. Poly-3-Hydroxybutyrate Production

Preliminary screening for the detection of bacterial isolates capable of poly-3-hydroxybutyrate biosynthesis and accumulation was performed using an analytical procedure previously used elsewhere by Zhang *et al.* [52]. Poly-3-hydroxybutyrate production was carried out in a nitrogen-deficient medium. Batch fermentation was carried out in a 250ml Erlenmeyer flask containing 100ml of culture medium. The flasks were inoculated and maintained at 30°C and 130 rotations per minute for 48, 96, 144 and 192 hours.

2.4. Poly-3-Hydroxybutyrate Extraction, Purification and Quantification

The isolated culture was employed for mass growth for 2-8 days in a rotary shaker at 37°C. The samples were centrifuged for 45 minutes at 6,000 rpm. The pellets were subsequently incubated at 60°C for 1 hour with sodium hypochlorite to break the cell walls of bacteria. Supernatant obtained was transferred to a Soxhlet system. Cell lipids and other molecules (except poly-3-hydroxybutyrate) were extracted by addition of 5mL of 96% (1:1 v/v) ethanol and acetone. Poly-3-hydroxybutyrate was extracted using chloroform. Chloroform extract was dried at 40ºC and 10 mL of concentrated sulfuric acid was added. They were heated at 100°C in a water bath for 20 minutes.

After cooling, quantification of biosynthesized poly-3-hydroxybutyrate was performed employing an analytical procedure previously used in other studies [2,53-55]. The biopolymer was quantified by UV Visible Spectrometry at 235nm using a Double Beam Optimal Geometry Genesys 10S Ultraviolet-Visible spectrophotometer (Thermo Scientific, USA) in comparison with a standard curve plotted between concentrations of crotonic acid and the corresponding absorbances at 235nm.

For dry cell weight (DCW) analysis, 10mL of culture sample was centrifuged at 11,200 × g for 20min. The cell pellet was washed twice with 1mL of distilled water and transferred to a dry petri dish. The pellet was dried to constant weight at 60°C to estimate the DCW in g/mL. Three independent replications were performed. The

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percentage of poly-3-hydroxybutyrate accumulated was estimated as the percentage composition of poly-3-hydroxybutyrate present in the DCW (measured in g/mL), which was calculated using equation (1):

$$\text{Poly-3-hydroxybutyrate Accretion} = \frac{\textbf{Dry weight of PHB}}{\textbf{Dry cell weight}} \times 100\% \qquad (1)$$

2.5. Characterization and Confirmation of the Extracted Polymer

Characterization and confirmation of poly-3-hydroxybutyrate recovered was done using crotonic acid assay. The powder was dissolved in sulphuric acid (1mg/mL) and heated at 100°C for 10 minutes to convert it into crotonic acid (brown colored). The solution was cooled, and its spectroscopic absorbance read at 260nm against a concentrated sulphuric acid as blank.

3. Experimental Results and Discussion

The average yields of poly-3-hydroxybutyrate from the cells are presented in **Table 1**.

Table 1. Poly-3-Hydroxybutyrate Yield in the Water Hyacinth Hydrolysate.

Incubation Time (Hours)	Recovered Poly-3-hydroxybutyrate (g/L)[a]	Poly-3-hydroxybutyrate Accretion (%)
48	1.2±0.0577	15.0
96	4.9±0.1155	61.3
144	4.2±0.0577	51.3
192	3.8±0.1155	47.5

a. Recovered poly-3-hydroxybutyrate is presented as Mean±Standard Error, S.E of triplicates.

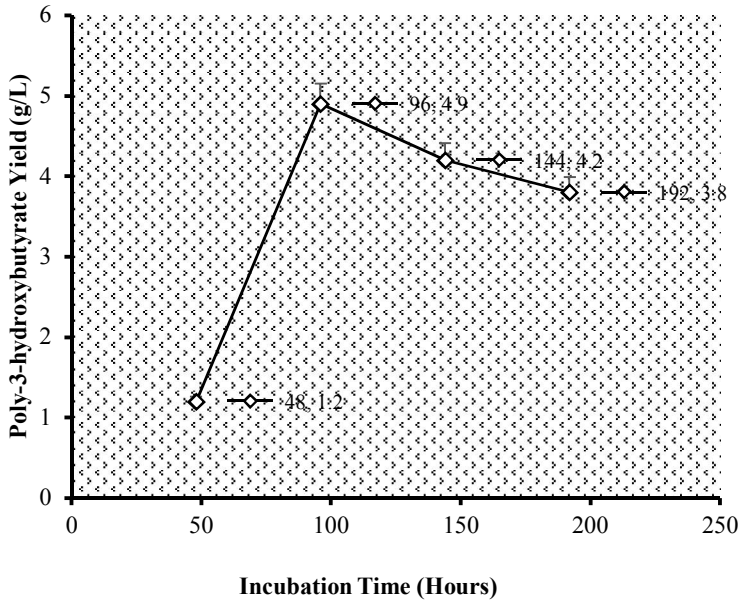

Figure 2. Poly-3-Hydroxybutyrate yield as a function of incubation time

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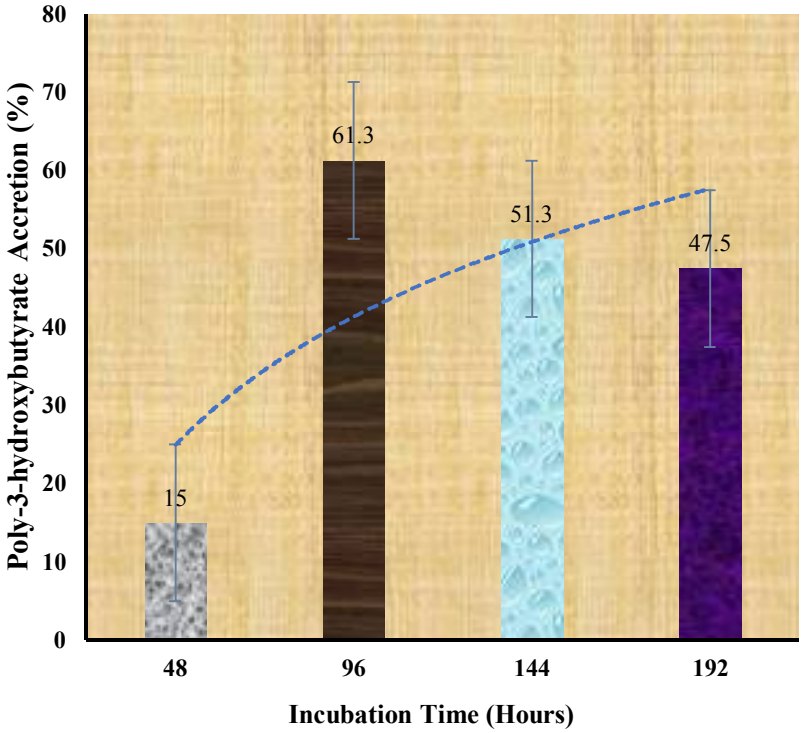

Figure 3. Poly-3-hydroxybutyrate accretion as a function of incubation time

3.1 Bacterial Staining

Utilization of the lipophilic stain to specifically stain the intracellular PHA granules accumulated by the sewage sludge isolated *B. megaterium* is a confirmatory swift routine approach for scrutinization of PHA biosynthesizing bacterial species [56]. The poly-3-hydroxybutyrate biosynthesizing colonies were bluish black and the poly-3-hydroxybutyrate granules were recognized by their affinity for the dye Sudan black, which is a presumptive test for presence of poly-3-hydroxybutyrate [57].

3.2 Hydrolysis of Water Hyacinth

The acid hydrolysis method of Pumiput *et al* [51] which was used while investigating lactic acid production from fruit waste registered success in hydrolyzing and harnessing reducing sugars from water hyacinth biomass in this study. This agrees well with the study of Preethi and co-authors [2].

3.3 Chloroform Extraction of Accumulated Poly-3-Hydroxybutyrate

Utilization of organic solvents such as chloroform for poly-3-hydroxybutyrate extraction is one of the most employed analytical procedures for recovering PHA and thus poly-3-hydroxybutyrate. 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 [58].

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3.4 Recovered Poly-3-hydroxybutyrate Yield and Accretion

There was gradual increase in poly-3-hydroxybutyrate biosynthesis by *B. megaterium* in the water hyacinth medium. A high yield of poly-3-hydroxybutyrate (4.9g/L, 61.3% poly-3-hydroxybutyrate) was realized on the fourth day of fermentation (96 hours) in water hyacinth medium (Table 1; Figure 2; Figure 3). The result of this investigation is corroborant with that observed with *Cupriavidus necator* [59] and *Pseudomonas aeruginosa* [2] where PHA yield was as high as 4.3g/L. Increase in the fermentation time in this study culminated in a significant decline ($p<0.05$) in poly-3-hydroxybutyrate biosynthesis. This could be correlated with the utilization of intracellular PHA granules as reserve food molecules during nutrient starvation [60]. Thus, it can be thought that until sporulation time, the bacteria produced poly-3-hydroxybutyrate 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. Bacterial spores are produced during the stationary phase as poly-3-hydroxybutyrate is being biosynthesized and utilized [61,62].

The results of this study is comparable to that of Klüttermann *et al* [63] who reported that *Agrobacterium radiobacter* gave a maximum accretion of 60% poly-3-hydroxybutyrate 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* [64] also reported that *B. megaterium* strain OU303A from sewage sludge successfully biosynthesized poly-3-hydroxybutyrate 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.

3.5 Quantification of Accretioned Poly-3-hydroxybutyrate

Polyhydroxyalkanoates can be chemically converted quantitatively to crotonic acid by heating in concentrated Sulphuric acid. The UV spectroscopic absorption maximum of crotonic acid is normally shifted to 260nm when concentrated sulphuric acid is used as the solvent [56]. Carboxyl compounds absorbs light below the UV range and hence are difficult to detect by spectrophotometry. 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 260nm [56]. The results of this pilot study confirmed that PHA, poly-3-hydroxybutyrate precursor was formed from fermented water hyacinth biomass, which is corroborant with the report of preceding authors [2, 65-67].

4. Conclusion

From this study, it was evidential that water hyacinth is a potential candidate for poly-3-hydroxybutyrate production in a batch fermenter. *Bacillus megaterium* successfully fermented the simple sugars in the water hyacinth hydrolysate. The yields of accumulated poly-3-hydroxybutyrate were generally greater for higher fermentation times; the maximum yield obtained was 61.3% per dry cell mass after 96 hours of fermentation.

Increase in fermentation time beyond 96 hours did not register any increment in poly-3-hydroxybutyrate yield. The utilization of water hyacinth as a starting substrate for poly-3-hydroxybutyrate biosynthesis using *B. megaterium* isolated from sewage could be a feasible strategy in managing the population of the noxious weed in the Victorian basin and the entire River Nile length. Further research should aim at screening to identify the strain of the bacterium harnessed from the sewage sludge as well as determine the nutritive parameters of the water hyacinth leaves.

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