Original Research Article

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

ABSTRACT

Aim: To assess the feasibility of utilizing water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laubach) as a potential carbon source for poly-3-hydroxybutyrate biosynthesis using *Bacillus megaterium* isolated from sewage sludge.

Study design: Water hyacinth was acid-catalysed and batch fermented for 144 hours in a nitrogen deficient medium using *Bacillus megaterium*.

Place and Duration of Study: The study was conducted using water hyacinth obtained from Lake Victoria, Kampala Uganda between October 2018 to March 2019.

Methodology: Poly-3-hydroxybutyrate biosynthesizing bacteria (*Bacillus megaterium*) was isolated from municipal sewage sludge and harnessed for fermentation of acid-catalysed water hyacinth biomass. Batch fermentation was carried out in 100 ml of culture media in a 250 ml fermenter for 48, 96, 144 and 192 hours to determine the best incubation time for maximum yield. Poly-3-hydroxybutyrate formed in the cytoplasm of the bacterial cells were extracted by chloroform extraction method and thereof confirmed and quantified by UV spectroscopy.

Results: An all-out net yield of 61.3% poly-3-hydroxybutyrate was realized after 96 hours of fermentation. Increase in fermentation time beyond 96 hours did not register any increment in poly-3-hydroxybutyrate yield.

Conclusion: 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. Further research should identify the strain of *Bacillus megaterium* from the sewage sludge as well as determine the nutritive parameters of water hyacinth leaves in Lake Victoria.

Keywords: batch fermentation, Lake Victoria, poly-3-hydroxybutyrate, sewage sludge.

1. 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) that is native to tropical America [1]. It is attested to have been introduced into East Africa as an ornamental verdure and progressed into Lake Victoria through the

Rwandese Kagera river [1]. The weed has flourished due to the available suitable growth conditions, total absence of omnivorous predators, elevated trace metal pollution and spacious environment of Lake Victoria. The plant is morphologically plastic, propagates prolifically and is highly flexible to variations in temperature, light intensity, water pH and salinity [1, 2]. Thus, it has a swift mode of propagation that enables 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]. The weed has since threatened the harvest of tilapia (*Oreochromis niloticus*), Nile perch (*Lates niloticus*) and silver fish (*Rastreneobola argentea*) [5]. Water hyacinth is a menace, impedes boat access, block communal water points along Victorian shorelines [3] and in prolific cases increase the 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 Lake Victoria, limiting growth of photosynthetic phytoplankton [1].

Although it is a deleterious aquatic weed, water hyacinth is an excellent source of biomass with an estimated hectare yield of 70,000 m^3 of biogas [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 which is far better than pure methane gas. Water hyacinth 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 and potassium in water [11-14]. The weed leaf concoction is phytotoxically effective against *Mimosa pigra* and *Vigna radiata* [15]. Water hyacinth is a substrate for the production of various furniture, handbags, ropes, potash, livestock feed for pigs [16], biofertilizers (compost or mulch) [17-19], paper manufacture [20], superabsorbent polymer material synthesis [21] and biosynthesis of poly-3-hydroxybutyrate [2, 22].

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 a high melting point and its biosynthesis is sustainable for voluminous production at economically reduced costs [23]. PHB is the simplest polyhydroxyalkanoate (PHA) with x and R in the general structure of PHAs -[O-CH(R)(CH2)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 it is gaining attention as a substitute for these plastics [24-26]. In addition, PHB has low permeability for water, molecular oxygen and carbon dioxide. PHBs are produced by a range of microbes cultured under different nutrient and environmental conditions [27]. The biopolyesters are harnessed as energy storage materials by the microbes in transient abundance of carbon with nutritional components such as phosphorous, magnesium, oxygen, nitrogen or sulfur being a limiting factor [28-34]. The biopolymers are naturally lipids, accumulates intercellularly as liquid, mobile or amorphous granules to ensure survival under physiological stress 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 and are fully biodegraded in microbe active environments within 5-6 weeks releasing carbon dioxide and water [35-37].

UNDER PEER REVIEW

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 [37].
Several studies have reported on PHB biosynthesis using various carbon sources. Yüksekdağ et al. [38] assessed the feasibility of PHB production by two *Bacillus* species (*B. subtilis* 25 and *B. megaterium* 12) in nutrient broth at various times between 6 and 48 hours. Their findings revealed that 0.101 g/L and 0.142 g/L of PHB with 18.03% and 14.79% yields after 45 hours were achieved with the two species respectively. After 48 hours, a significant reduction in PHB yield was observed. Whereas PHB accumulation in the culture broth by the strains were nearly insignificant with the two carbon and nitrogen sources, the highest PHB level was noted in protease peptone enriched medium. In this enriched broth, PHB yield of *B. subtilis* 25 was 78.69% whereas in the same nitrogen source *B. megaterium* 12 had 77% yield. In another 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 [39]. 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 formation rate of PHB up to 0.016 hr^{-1} and specific growth rate of up to 0.25 hr^{-1} were reported during the experimental growth. The chemical structure and thermal properties of PHB produced from MOL and CSL were comparable to that of the commercial PHB except for the significantly higher molecular mass (about 3.9×10^6 Da) and lower degree of crystallinity.

The effect of various carbonaceous and nitrogenous sources on PHB production was investigated by Gouda et al [40]. The highest yield 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 PHB achieved with 2% molasses. 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 the 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 [41]. Its probable ability to be utilized in PHB production 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 carbon-13 Nuclear Magnetic Resonance and Fourier Transform Infrared analyses, they concluded that despite the sporulation phenomenon exhibited by the novel *Bacillus* strain, its intracellular PHB biosynthesis potential was higher than those previously reported in literature. A study conducted by Rodrıguez-Contreras et al [42] 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 [43, 44]. More so, the industrial scale conditions utilize conventional medium and moderate salt content; an environment that was already replicated in their previous study [42] and carried on in the aforeacknowledged study.

UNDER PEER REVIEW

Unfortunately, production of PHB 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, 45] and potentially cheap substrates. The genus *Bacillus* received attention [46] due to the stability of its replication and plasmids maintenance. Many *Bacillus* species have reported PHB production potential [47, 48]. This study reported the feasibility of utilizing the pleustophytic ecological plague (*Eichhornia crassipes* (Mart.) Solms-Laubach in Lake Victoria, Uganda for batch biosynthesis of PHB using *B. megaterium* isolated from municipal sewage sludge.

2. MATERIAL 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 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 carbon source. Sewage sludge samples (5 kg) were collected from Lubigi Sewage and Faecal Sludge Treatment Plant (SFSTP). The treatment plant, operated by National Water and Sewerage Corporation (NWSC) in 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 400 m^3 per day capacity for faecal sludge treatment [49]. Sludge samples were collected 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 was isolated from sewage sludge, purified and identified as *B. megaterium* following standard methods [50]. The purified isolates were cross streaked on nutrient broth (2.5g/L peptone, 2.5g/L NaCl, 1.0g/L yeast extract and 0.5g/L beef extract). 100 ml 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.

Substrate hydrolysate preparation was performed following a modified analytical procedure advanced by Pumiput *et al* [51]. Aliquots (8.0 ± 0.1 g) of powdered water hyacinth leaves was steam exploded in an autoclave at 121°C for 20 minutes. 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 minutes 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 121°C with 1% hydrochloric acid (v/v) for 30 minutes. 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 [2, 51].

2.3 PHB production, extraction, purification and quantification

Preliminary screening for the detection of bacterial isolates capable of PHB biosynthesis and accumulation was performed using the analytical procedure used by Zhang et al. [52]. PHB

UNDER PEER REVIEW

production was carried out in a nitrogen-deficient medium. Batch fermentation was carried out in a 250 ml Erlenmeyer flask containing 100 ml of culture medium. The flasks were inoculated and maintained at 30°C and 130 rpm for 48, 96, 144 and 192 hours.
The isolated culture was employed for mass growth for 2-8 days in a rotary shaker at 37°C. For analysis, 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. Supernatants obtained were transferred to a Soxhlet system. Cell lipids and other molecules (except PHB) were extracted by addition of 5 mL of 96% (1:1 v/v) ethanol and acetone. PHB was extracted using chloroform, dried at 40ºC followed by addition of 10 mL of concentrated sulfuric acid. The resultant solution was subsequently heated in a serological water bath at 100°C for 20 minutes.
After cooling, quantification of biosynthesized PHB was performed employing an analytical procedure used by previous studies [2, 53-55]. Briefly, 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 minutes. 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 60°C to estimate the DCW in g/mL. Three independent replications were performed and the percentage of PHB accumulated was estimated as its percentage composition in the DCW measured in g/mL (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 100°C for 10 minutes to convert it into crotonic acid (brown colored). 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 in this study 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 (Marshall Scientific, Hampton, NH, USA) was used for all analytical weighing. 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. All experiments were performed in triplicate and results presented as means with standard errors attached.

2.6 Statistical analysis of results

Results from the triplicate analyses were subjected to statistical evaluation. One-way analysis of variance was done followed by Turkey pairwise test to separate the

UNDER PEER REVIEW

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) [56].

3. RESULTS AND DISCUSSION

3.1 Bacterial staining and hydrolysis of water hyacinth

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 [57]. The PHB biosynthesizing colonies were bluish black and the PHB granules were recognized by their affinity for Sudan black dye, which is a presumptive test for presence of PHB [58]. 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 agreed well with the study of Preethi et al. [2].

3.2 Recovered poly-3-hydroxybutyrate and its accretion

Utilization 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 [59]. The average yields of PHB from the cells are presented in **Table 1** and illustrated in Figure 1 and Figure 2.

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

** Recovered PHB is presented as Mean ± Standard Error, S.E of triplicates.*

UNDER PEER REVIEW

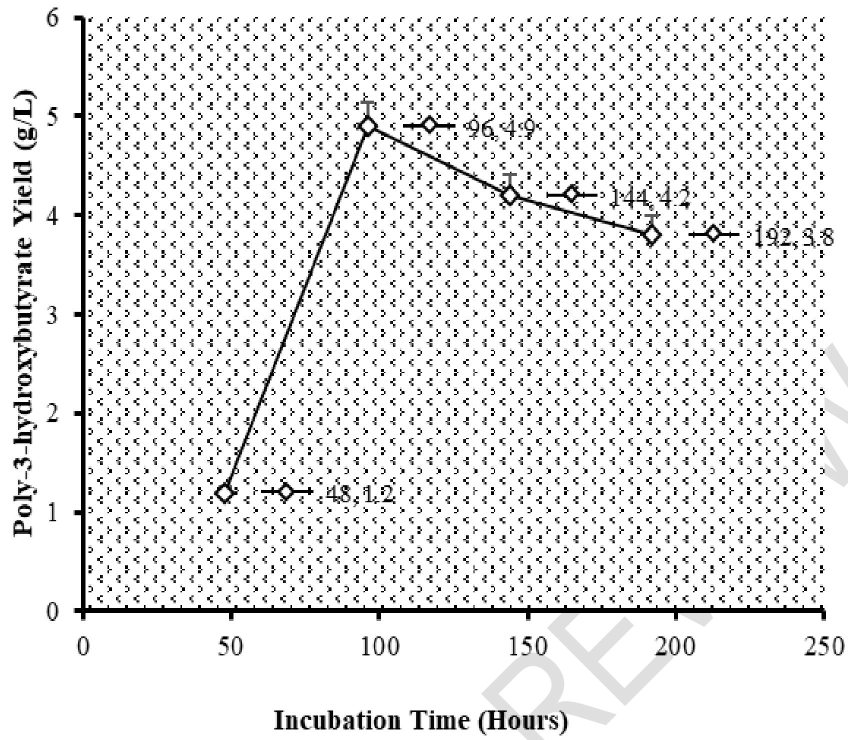

Fig. 1. Poly-3-Hydroxybutyrate yield as a function of incubation time

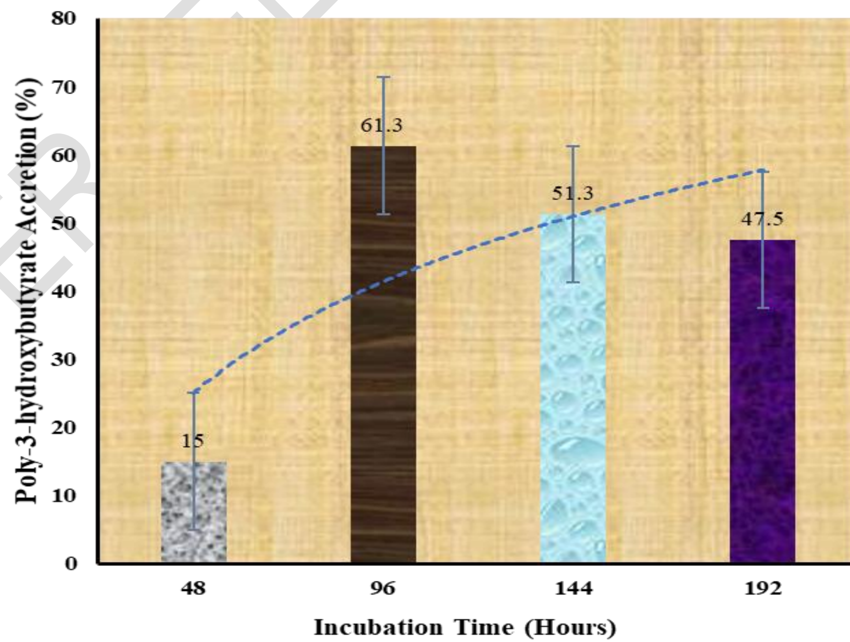

Fig. 2. Poly-3-hydroxybutyrate accretion as a function of incubation time

UNDER PEER REVIEW

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 (Figure 1, Figure 2). The result of this investigation is corroborant with that observed with *Cupriavidus necator* [60] and *Pseudomonas aeruginosa* [2] where PHA yield was 4.3 g/L. Increase in the fermentation time in this study culminated 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 [61]. 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. Bacterial spores are produced during the stationary phase as PHB is being biosynthesized and utilized [62, 63]. The results of this study is comparable to that of Klüttermann *et al* [64] 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* [65] 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 quantitatively 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 [57]. 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 260 nm [57]. The results of this pilot study confirmed that PHA, a PHB precursor was formed from fermented water hyacinth biomass, which is corroborant with the report of preceding authors [2, 66-68].

UNDER PEER REVIEW

4. CONCLUSIONS AND RECOMMENDATIONS

From this study, it was evidential that water hyacinth is a potential candidate for PHB production in a batch fermenter. *Bacillus megaterium* successfully fermented the simple sugars in the water hyacinth hydrolysate. The yields of accumulated PHB 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 PHB yield. The utilization of water hyacinth as a starting substrate for PHB 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 identify the strain of the bacterium harnessed from the sewage sludge as well as determine the nutritive parameters of the water hyacinth leaves.

COMPETING INTERESTS DISCLAIMER:
Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use 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.

UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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