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Water Hyacinth: A Potential Substrate for Bioplastic (PHA) Production Using *Pseudomonas aeruginosa*

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Abstract
Water hyacinth (*Eichhornia crassipes)* regarded as the world's worst weed has potential nutritional status that exploits its use as substrate for commercial production of value added products. This research work focuses on utilizing a cosmopolitan aquatic weed water hyacinth as a potential substrate for the production of PHA using *Pseudomonas aeruginosa* as the fermenting organism. Acid hydrolysis using HCl (1%) was used for breaking down complex sugars in the water hyacinth hydrolysate to easily fermentable reducing sugars. Sodium Hypochlorite digestion was employed for cell lyses and subsequent release of the intracellular PHA content from *Pseudomonas aeruginosa*. Extraction of PHA from the fermentation media using chloroform extraction method produced a net yield of 65.51 % on 72 hours of incubation. Preliminary confirmation of the recovered product was done using Thin Layer Chromatography and Crotonic Acid Assay. Detailed study of the functional groups present in the recovered product was done using FTIR.

Keywords: Polyhydroxyalkanoates, Water hyacinth, Hydrolysate, Crotonic acid, FTIR.

Introduction
The very versatile nature of petroleum based plastics had made it as an inevitable part in every sphere of human life. In spite of their potential applications, the recalcitrant nature of petroleum based plastics made the pressure on environment to get worse than ever before. Thus a new approach to produce bio based polymers and composites as an alternative to conventional plastics was found to be the only effective method to ensure degradability and environmental friendly approach. Polyhydroxyalkanoates (PHAs) are a family of biopolyesters synthesized and accumulated by a wide range of microorganisms as reserve food material. These are the only plastics produced exclusively by microorganisms and hence are completely degraded to benign compounds (Anderson and Dawes, 1990) [1]. Polyhydroxyalkanoates are hydroxyalkanoic acids linked to each other by an ester linkage. Polyhydroxybutyrate (PHB) is the most commonly occurring and hence most studied PHA. The composition of the synthesized polymer is influenced by the bacterial strain as well as type and relative quantity and quality of carbon sources supplied to the growth medium (Steinbuchel *et al.,* 2003) [2]. The major factor restricting the commercial production of polyhydroxyalkanoates is the cost of production of these biopolymers. Nowadays more research works focuses on tuning agro industrial waste as raw material for biopolymer production.

Water hyacinth (*Eichhornia crassipes)* is a pleustophytic hydatophyte, a cosmopolitan aquatic weed; it is widely recognized as the world's worst aquatic weed. This weed forms dense impenetrable mats across water surface, limiting access by man, animals and machinery. Moreover, navigation and fishing are obstructed, and irrigation as well as drainage systems become blocked. They can tolerate a wide range of environmental conditions such as temperature, illumination, pH, salinity, wind, current and drought. The plant is morphologically very plastic with a rapid mode of vegetative propagation which makes it well adapted to long distance dispersal and successful colonization of diverse ecological niches. It is one of the most prolific aquatic plants which spread at an alarming rate having spikes of large blue flowers and roundish leaves with inflated bladder - like petioles. In spite of its environmental deteriorating effects the weed still offers potential to be used as substrate for production of commercially important products because of its promising carbohydrate content.

2. Materials and Methods

2.1. Collection of raw material

Water hyacinth leaves were collected from 'Shokanashini' river, Palakkad, Kerala. It was washed with water several times for the removal of dirt and dried in hot air oven at 70 ˚C for 48hrs and then grinded into fine powder and stored in air tight container in room temperature until further analysis.

2.2. Analysis of nutritive parameters in water hyacinth

Moisture content and Crude fiber content of Water hyacinth leaves were analyzed using standard A.P.H.A, 2005 [3] protocol. Lipid content and crude fiber content of the leaves were analyzed using A.O.A.C, 2005 [4] method. Protein content of the water hyacinth leaves were assessed using Lowry's *et al*., [5] method. The total sugar content after hydrolysate preparation was done using Anthrone method (Hedge *et al.,* 1962) [6]. The reducing sugar content post acid hydrolysis was analyzed using DNS method (Miller, 1959) [7].

2.3. Inoculum preparation

Pseudomonas aeruginosa culture war procured from MTCC Chandigarh and maintained in Kings B medium. This was used as inoculum for further fermentation studies.

2.4. Screening for PHA production

Qualitative test (Sudan Black staining)

Bacterial test culture was smeared on a clean glass slide and it was heat fixed. A few drops of Sudan black B solution (0.02gm in 100ml ethanol) was added to the smear and left for 10 minutes. Then the slides were washed gently with ethanol and counter stained with safranin and allowed to dry. The slide was observed under oil immersion under optical microscope (John and Ralph, 1961) [8].

Quantitative test (Chloroform precipitation test)

50mL of 24hrs old culture of *Pseudomonas* in nutrient broth medium was centrifuged at 6000rpm for 20 minutes. The pellets were then suspended in 5mL sterile water and vortexed for 8 minutes. To 2mL of cell suspension, 2mL of 2N HCL was added and heated to boiling temperature for 2hrs in a water bath. The tubes were then centrifuged at 6000rpm for 20 minutes. To the supernatant 5mL of chloroform was added. The tubes were left overnight in a shaker, after 24hrs the content of the tubes was poured onto plates and allowed to dry out (Doi *et al*., 1995) [9].

2.5. Preparation of hydrolysates for fermentation

Steam explosion

The modified method of Pumiput *et al.,* (2008) [10] was used for substrate hydrolysate preparation. About 8gram of dried powder of water hyacinth leaves was steam exploded in an autoclave at 121°C for 20min.Sterile water was added to the wet pretreated material to make the volume of 200ml and boiled at 80°C for 30 min. Later the hydrolysate was recovered by filtration with cheese cloth.

Acid hydrolysis

Acid post hydrolysis of hydrolysate was carried out to cleave the oligosaccharides in to monomeric sugars by autoclaving at 121°C with concentration of 1% HCl v/v for 30min (Pumiput, 2008) [10].

pH adjustment

The hydrolysate from acid post hydrolysis was adjusted with NaOH to pH 6-6.8 and the precipitate was removed by filtration with whatman filter paper No.1 (Pumiput, 2008) [10].

2.6. Fermentation conditions

Modified nutrient broth media comprised of the following; Glucose (10gm), Peptone (5gm), Yeast Extract (3gm), NaCl (5gm) in water hyacinth hydrolysate (1000ml) was prepared and used as fermentation medium for bioplastic production (Radha *et al*., 2014) [11]. The pH of the medium was adjusted to 7 and inoculated with 10 % of overnight culture of *Pseudomonas.* The flask was incubated at 37 ˚C in a shaking incubator for 96hrs. Modified nutrient broth medium containing 1% glucose prepared in distilled water was used as comparative standard. This is followed by extraction of PHB from culture broth.

2.7. Extraction of PHA

The extraction of PHA was done using the method of Santhanam and Sasidharan (2010) [12] using the solvent chloroform. After the incubation period of the fermented media was centrifuged at 10,000rpm for 15min.The supernatant was discarded leaving the pellet, which was air dried and weighed. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37 ˚C for 1-2h for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged again. Finally PHA granules in the sediment were washed twice with acetone and diethyl ether (1:1). The resultant polymer granules were dissolved in boiling chloroform and air dried to obtain PHA powder.

2.8. Analytical procedure

The bacterial cultures were centrifuged at 6,000rpm for 5min to obtain the cell pellet. The cell pellet was dried to estimate the wet cell weight (DCW in g/mL) (Du *et al*., 2001) [13]. Residual biomass, PHA accumulation and concentration of PHB (Zakaria *et al*., 2010) [14] was calculated by the following formulae:

Residual biomass (g/mL) =Wet Cell Weight (g/mL) - Dry weight of extracted PHA (g/mL)

$$\text{PHA Accumulation (\%)} = \frac{\text{Dry weight of PHA extracted (g/mL)}}{\text{WCW (g/mL)}} \times 100$$

2.9. Confirmation of PHA

TLC

TLC was performed on Silica Gel Plate. An aliquot of sample (PHA dissolved in methanol) was spotted on silica gel plate. The developing Solvent system used is Benzene: Ethylacetate (1:1). The spots were visualized using iodine chamber (Rawate *et al.,* 2008) [15].

Crotonic acid assay

The PHA powder was dissolved in sulphuric acid (1mg/mL) and heated at 100 ˚C for 10 minutes to convert PHA into crotonic acid, which was brown coloured. The solution was cooled and the absorbance was read at 260nm against a concentrated sulphuric acid as blank in spectrophotometer. A standard curve was prepared with pure PHA concentration ranging from 20-100µg/mL (Law and Slepecky, 1969) [16]. The quantity of PHA produced was determined by comparison with the standard.

FTIR

Infrared spectra (IR) were also used to identify the functional groups present in the bioplastics. 1mg of dry sample was mixed with 100mg of dry KBr and the compressed to prepare salt – disk (3mm diameter). These disks were analysed under Fourier Transform IR- Spectrophotometer. The frequencies of different components present in active sample were analysed. The absorption was read between 400-4000 cm^{-1} (Oliveira *et al*., 2007 [17] and Pandiyan *et al*., 2010 [18]).

3. Results

3.1. Analysis of nutritive parameters in water hyacinth

Nutritive composition of water hyacinth leaves were tabulated (Table 1)

Table 1: Nutritive parameters in Water hyacinth leaves

Serial No.	Nutritive Parameters	Obtained Value (%)
1.	Moisture Content	86.3 ± 0.21
2.	Ash Content	16.5 ± 0.32
3.	Protein Content	15.3 ± 0.12
4.	Fiber Content	15.9 ± 0.29
5.	Lipid Content	1.6 ± 0.04
7.	Total Sugars	32.1 ± 0.06
8.	Reducing Sugars	19.8 ± 0.14

Data represents the mean of triplicates ± standard deviation

3.2. Screening for PHA production

Qualitative test (Sudan Black staining)

Pseudomonas aeruginosa culture stained with lipophilic stain Sudan black when viewed under microscope exhibited dark intracellular granules in pink coloured cells.

Quantitative test (Chloroform precipitation test)

White colored precipitates were formed on the petriplate due to the precipitation of PHA granules (Figure 1) dissolved in chloroform, after the evaporation. This confirms the PHA production ability of the test culture.

Fig 1: Choloroform precipitation test

3.3. Preparation of hydrolysates for fermentation

The high amount of carbohydrates present in the plant leaves were extracted in to the hydrolysate (Figure 2) through steam explosion method as suggested by Puimput *et al.,* (2008) [10]. Acid hydrolysis with concentrated HCl (1%) was done to convert complex plant sugars in to easily fermentable monomeric (Figure 3) residues.

Fig 2: Water hyacinth hydrolysate

Fig 3: Acid hydrolysate

3.4. Analytical procedure

Irrespective of the type of media used for fermentation, the PHA production as directly proportional to bacterial cell density. The maximum amount of PHA accumulation was found on the 3rd day of fermentation on both the medium. The biomass produced, PHA content and percentage of PHA accumulation were tabulated (Table 2).

Table 2: Biomass, PHA weight and PHA content (%) in MNB and WHM

Incubation Time (hrs)	Modified Nutrient Broth (MNB)			Water Hyacinth Medium (WHM)		
	Biomass (g/L)	PHA (g/L)	PHA content (%)	Biomass (g/L)	PHA (g/L)	PHA content (%)
24	1.1 ± 0.02	0.3 ± 0.09	35.57	1.4 ± 0.01	0.4 ± 0.01	36.16
48	2.7 ± 0.12	1.1 ± 0.08	43.64	3.9 ± 0.02	1.7 ± 0.05	44.56
72	4.2 ± 0.03	2.2 ± 0.07	54.10	4.2 ± 0.09	2.8 ± 0.01	65.51
96	4.3 ± 0.26	2.1 ± 0.08	51.51	4.1 ± 0.10	2.6 ± 0.08	65.14

Data represents the mean of triplicates ± standard deviation

The highest PHA content was observed with water hyacinth medium (65.51%) and modified nutrient broth (54.10%) during third day of fermentation.

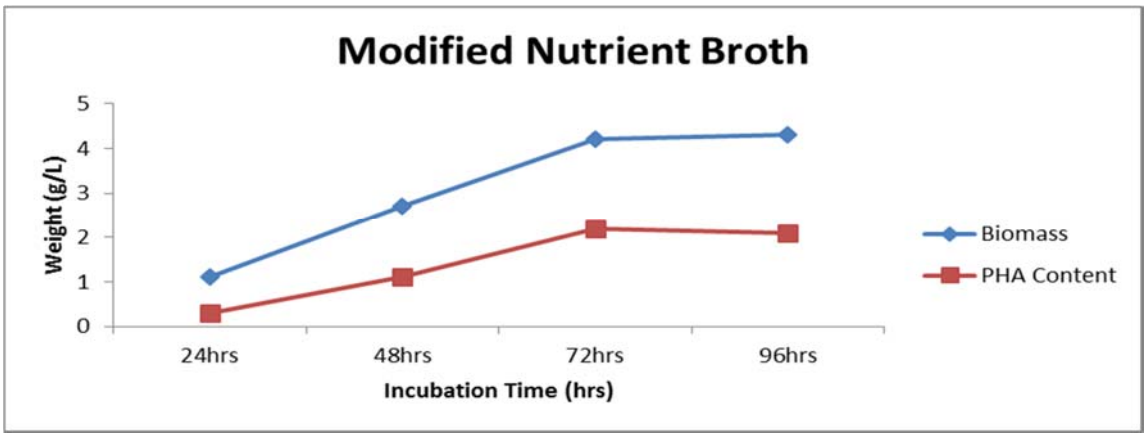

Fig 4: Biomass and PHA content in Modified Nutrient Broth (MNB)

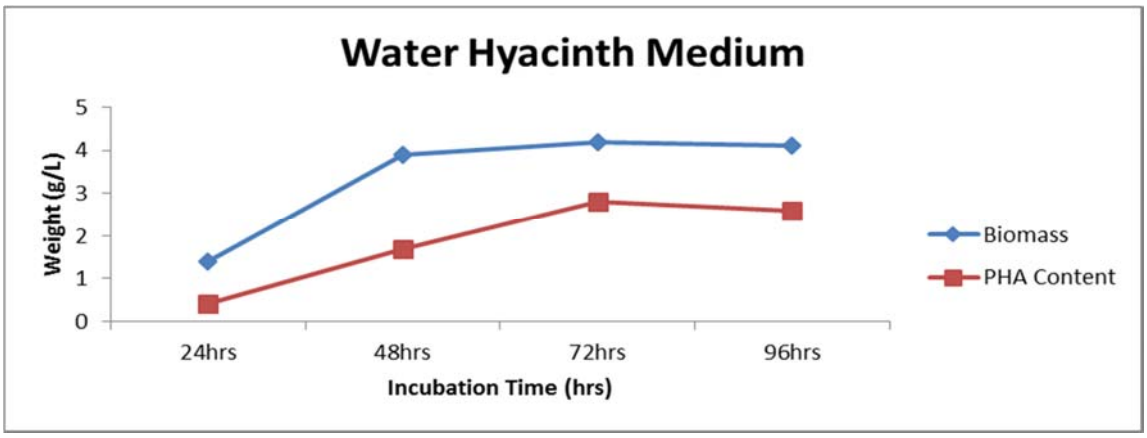

Fig 5: Biomass and PHA content in Water Hyacinth Medium (WHM)

3.5. Confirmation of PHA

The extracted PHA granules were dissolved in minimum amount of Benzene: Ethyl acetate mixture and loaded on to silica gel TLC plates. Upon exposure to iodine vapors yellowish brown precipitates were formed in TLC. This is similar to the results observed by previous investigators. The crotonic acid assay for quantification of PHA recovered from fermentation broth revealed a PHA composition of 97µg/mL and 113µg/mL for Modified nutrient broth and Water hyacinth medium respectively. Evaluation of functional group present in the PHA recovered from water hyacinth medium was done using FTIR spectroscopy (Figure 6).

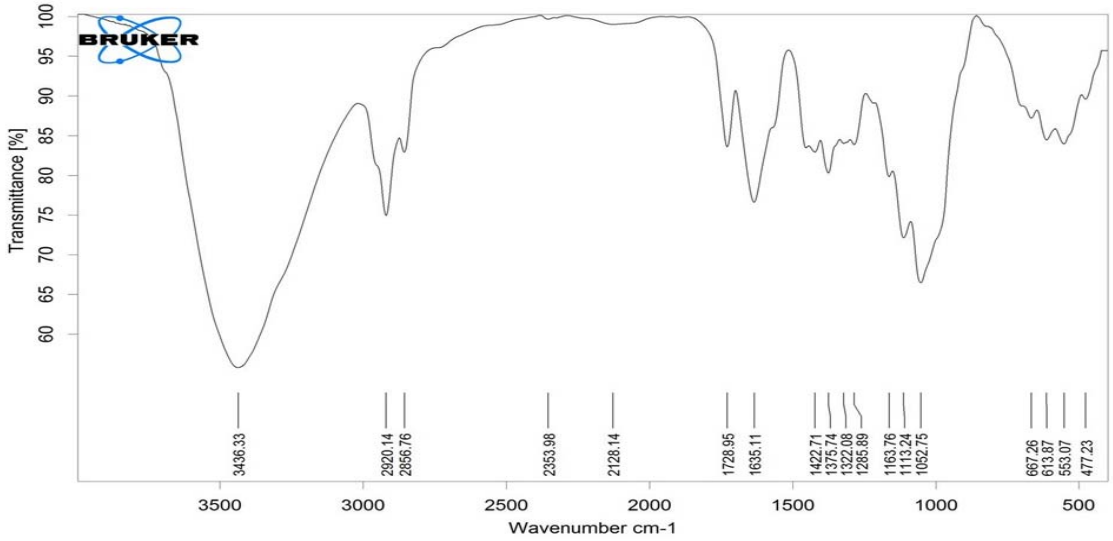

Fig 6: FTIR spectrum of extracted PHA molecule from water hyacinth medium

The FTIR spectrum confirms the product is in fact a bioplastic according to standard IR spectra. The peak at 3436.33 cm^{-1} indicates a strong bond of H stretching originated by terminal OH group. The peaks at 2920.14 cm^{-1} and 2856.76 cm^{-1} corresponds to aliphatic C-H stretching originated from the alkane groups. The peaks at 1728.95 cm^{-1} and 1635.51 cm^{-1} indicates a weak C=O stretching for the conjugated carbonyl or amide group. The peaks at 1422.71 cm^{-1} and 1375.74cm^{-1} corresponds to C-H bond contributed by alkanes. The bond at 1113.24 cm^{-1} corresponds for C-O stretching. The peaks at 667.26 cm^{-1}, and 613.87 cm^{-1} corresponds to alkyl halides.

4. Discussion

The use of lipophilic stain Sudan Black to specifically stain the intracellular PHA granules accumulated by bacteria was a routine and rapid method used for screening of PHA producing organism (Huisman, 1982) [19]. The use of solvents like chloroform for PHA extractions was one of the oldest methods of PHA recovery. Chloroform acts by altering the cell membrane permeability followed by solubilizing the PHA component (Chen *et al.,* 1994) [20]. Analyses of nutritive parameters in water hyacinth leaves were in correlation with the results obtained by previous researchers. The high moisture content in the leaves could be attributed to their constant contact with water in lakes and ponds. In a similar study by Little (1979) [21] a moisture content of about 80.95% was observed. The crude protein content in the present study was observed to be higher than that recorded by Chalmer (1968) [22]. The appreciable amount of lipids found in the leaves was assumed to be the product of photosynthesis and was in favorable agreement with that of Hossain (1959) [23]. The acid hydrolysis method suggested by Pumiput *et al*., (2008) [10] working with lactic acid production from fruit waste was successful in producing and extracting reducing sugars from water hyacinth.

The high amount of PHA produced during the third day of fermentation in water hyacinth medium supplemented with glucose highlighted the importance of C: N ratio in PHA production. Nutrient limitation and incorporation of complex nitrogen sources along with simple absorbable reducing sugars in medium could further enhance the PHA productivity. The result of the present study was in correlation with that observed with *C.nectar* (Murugesan *et al*., 2012) [24] were PHA productivity as high as 4.3g/L as achieved. The maximum productivity was achieved during 72 hours of incubation. Further increase in the incubation period resulted in the decline of PHA content. This could be correlated with the utilization of intracellular PHA granules as reserve food molecules during nutrient starvation as previously explained by Grothe *et al*., (1999) [25].

The application of Thin Layer Chromatography was used as a routine work for primary characterization of PHA molecule. The appearance of brownish black spots when exposed to iodine vapor correlated with the study of Rawate *et al*., (2002) [15]. PHA can be converted quantitatively to crotonic acid by heating in concentrated H_2SO_4 and the UV absorbtion maximum of crotonic acid is shifted to 260nm when concentrated sulphuric acid is the solvent (Law and Slepecky, 1961) [16] carboxyl compounds absorbs light below the UV range and hence are difficult to detect by spectrophotometry. The actual principle of crotonic acid assay reveals in the fact that the UV absorption maximum of α,β unsaturated acids undergoes a strong bathochromic shift or shift to lower frequency in sulphuric acid and can be recorded in the UV range. The absorption maximum shifts to 260nm (Slepecky & Law, 1961) [16]. The extracted PHA was routinely analyzed for identification of its functional groups through FTIR analysis. In FTIR spectra the deep peaks (pointing downwards) corresponds to the functional groups and indicates the nature of molecule. Also, the intensities are measured for both stretching as well as bending. However, stretching gives a spectrum of higher intensity, making it easier and clearer to study. The results obtained in the present investigation confirmed the product to be PHA and is in complete agreement with the results obtained by previous investigators (Hernandez *et al*., 2010 [26]; Sandhya *et al*., 2012 [27]; Chen *et al.,* 2009 [28]).

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