Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3 (1): 33-37

Received: 11-12-2014
Accepted: 25-12-2014

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Screening and optimization of Poly Hydroxy Butyrate (PHB), using *Eichhornia crassipes* as substrate by *Bacillus cereus* and *Bacillus subtilis*

Raheleh Mehrabi, Gholamreza Bagheri, Farhad Alipour

Abstract
Eichhornia crassipes or water hyacinth-derived sugar molecules can be converted into PHB, a material for making biodegradable plastic. Making PHB from natural resources can reduce cost and harmful gas emissions. In our research PHB production was done by Solid Surface Fermentation and Submerge Fermentation technics for *Bacillus cereus* and *Bacillus subtilis* using *Eichhornia crassipes* as a substrate. The production of PHB was optimized at different incubation time, pH and N_2 rate source. The higher production of PHB for *B. subtilis* was obtained by Submerge Fermentation and for *Bacillus cereus* by Solid Surface Fermentation. The present study proves that *E. crassipes* can be applied for PHB production at the presence of *B. cereus* and *B. subtilis*.

Keywords: Polyhydroxybutyrate, *Eichhornia crassipes*, *Bacillus cereus*, *Bacillus subtilis.*

1. Introduction
Economic growth and changing consumption and production patterns are resulting in rapid increase in the generation of waste plastics in the world [1]. This implies that on one hand, more sources are being used to meet the increased demand of plastic, and on the other hand, more plastic waste is being generated. Due to the increase in generation, waste plastics are becoming a major stream in solid waste. This increase has turned into a major challenge for local authorities, responsible for solid waste management and sanitation [2]. The production and use of bioplastics are generally regarded as a more sustainable activity when compared with plastic production from petroleum (petroplastic), because it relies less on fossil fuel as a carbon source and also introduces fewer, net-new greenhouse emission if it biodegrades. They significantly reduce hazardous waste caused by oil-derived plastics [3], which remain solid for hundreds of years, and open a new era in packing technology and industry.
PHB was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne [4]. PHB is a polymer belonging to the polyesters class that are of interest as bio-derived and biodegradable plastics. Microbial biosynthesis of PHB start with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA, which is subsequently reduced to hydroxybutyryl-CoA [5]. Hydroxybutyryl-coA is then used as a monomer to polymerize PHB. PHB has attracted much commercial interest as a plastic material because its physical properties are remarkably similar to those of polypropylene (PP), even though the two polymers have quite different chemical structures. Most importantly, PHB is rapidly biodegradable, unlike PolyPropylene. Two major factors inhibiting the widespread use of PHB lie in its production costs, which are a lot higher than plastics produced from petrochemicals, since PHB as it is currently produced cannot handle high impact. Because PHB is both biodegradable and nontoxic it is an excellent material to use for medical purposes.
E. crassipes degrades water quality by blocking photosynthesis [6]. This creates a cascading effect by reducing other underwater life. Scientists have now shown that the infamous weed can be used to make biodegradable plastic. Water hyacinth-derived sugar molecules can be converted PHB, a polymer that is a raw material for making biodegradable plastic [7]. Regarding the high cost of carbon source which is needed to produce PHB by bacteria we selected *E. crassipes* which is worldwide aquatic waste, but also it is a good carbon source can be used as substrate for PHB production.

Aim of the current study is production and optimization of PHB by *B. cereus* and *B. subtilis*, and comparison of the results of PHB concentration by *B. cereus* and *B. subtilis*.

2. Material and Method

The study has been done in Department of agriculture, University of Pune, Pune, India; within Jun 2013 to august 2014.

2.1 Producer strain: The pure culture of *B. cereus* and *B. subtilis* were procured from Mitcon Biopharma Laboratory, Pune. The culture was preserved on a nutrient agar media slant at 4 ^{0}C in refrigerator. Screening for PHB production by *B. cereus* and *B. subtilis*; the screening was done by Sudan Black B staining and the Safranin solution to conformation PHB granules produced in *B. cereus* and *B. subtilis*.

2.2 Substrate preparation: Fresh water plants with long stem were collected from Mutha River, near Corporation Bridge, Pune. Collected *E. crassipes* were washed to remove adhering dirt and chopped in small pieces and ground it. Then powder was prepared after drying it in hot air oven at 100 °C for 24 hrs.

2.3 Media preparation with substrate: Solid surface fermentation: (10%) *E. crassipes* powder was autoclaved without basal media.

2.4 Submerge fermentation: (10%) *Eichhornia* powder was boiled in distilled water till its volume become 1/3rd of its actual volume then filtered it with muslin cloth and basal media was added to obtain the first volume [8].

2.5 Inoculum preparation: For all of the experiments, the inoculum was prepared in 250 mL Erlenmeyer flask containing 50 mL of sterile nutrient broth. The flasks were incubated at 37 °C for 24 hrs. On a rotary shaker at 120 rpm [9]. After incubation 10 ml cultural broth of 24 hrs *B. cereus* and 10 ml culture broth of *B. subtilis* was centrifuged at 5000 × g for 15 min. The pellet was inoculated in modified Basal media. Inoculated production media for solid surface fermentation was kept at 37 °C for 48 hrs, and for submerge fermentation was at 37 °C for 48 hrs at 220 rpm on a rotary. Production of PHB using *Eichhornia* powder as a substrate involved two kinds of fermentation; Solid surface fermentation and Submerge fermentation. After incubation the active culture of both bacteria was separately inoculated into two flasks containing different kinds of fermentation composition; solid surface fermentation and submerge fermentation with basal media composition. Composition of basal media (without distilled water) plus 5% *E. Crassipes* hydrolysate (as glucose source) was subjected to submerge fermentation and composition of basal media plus 5% *E. Crassipes* fine powder (as a carbon source) was subjected to solid surface fermentation; considering in which kind of fermentation PHB yield would be higher for each bacteria. Incubation time was followed for 48 hrs.

2.6 PHB extraction: After incubation, each sample was centrifuged for 15 min at 5000 rpm. For Solid surface fermentation the sample was filtered with Muslin cloth. But for submerge fermentation the sample was poured into a centrifuge tube as such [10]. The pellet was oven dried at 50 ^{0}C for 12 hours. The total dry weight was determined for further procedure. The next day Sodium hypochlorite was added to dry cell mass and was incubated at room temperature for 2:30 hrs. This sample was centrifuged at 5000 rpm For 15 minutes and the supernatant was used for further treatment. Using 96% v/v ethanol: acetone (1:1) cell lipid and other molecules, except PHB were extracted from the supernatant. The PHB extraction was done by a hot chloroform method (adding chloroform to the tube containing supernatant and ethanol: acetone in water bath). The solution was poured into clean petri plates. The PHB crystals were obtained after evaporation of chloroform (Fig. 1).

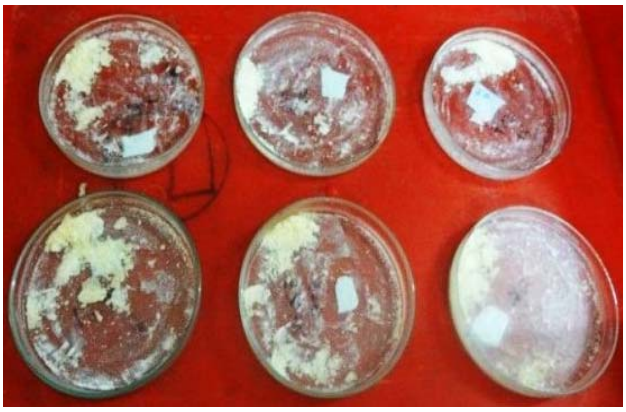

Fig 1: Crystals of PHB extract

2.7 Determination of PHB content: PHB crystals underwent dehydration on treatment with sulfuric acid and heat, to crotonic acid. The extracted PHB was converted to crotonic acid by adding 98% sulfuric acid and heating to 100 ^{0}C for 10 min. Crotonic acid shows maximum absorption at 235 nm in a UV spectrophotometer against a sulfuric acid as blank. The amount of PHB per gram dry weight of bacterial cells was determined form standard graph of crotonic acid [11].

2.8 Crotonic acid method: 0.1 g of extracted PHB was transferred to a clean test tube. 10 ml of sulfuric acid was added. It was boiled on hot water bath for 10 min. Then it was allowed for cooling at room temperature for 30 min. Absorbance of the solution was measured at 235 nm against sulfuric acid blank [12].

2.9 Media optimization: The media were optimized by following parameters and then PHB production was tested; The media were optimized at different incubation time i.e. 24 hrs. 48 hrs. and 72 hrs. The media were optimized at different pH i.e. 5, 7.2 and 9. The media were optimized at different percentages of Nitrogen source i.e. 0.25%, 0.50% and 1%.

3. Results

3.1 Sudan black B staining to conform production of PHB in *B. cereus and B. subtilis*:

Sudan black b staining showed the ability of *B. cereus* and *B. subtilis* to produce PHB. The PHB granules were recognized by their affinity for the dye Sudan black, which is a presumptive test for presence of PHB. The stained preparations were examined under a compound microscope with an oil immersion lens for determining cellular PHB accumulation. The production of PHB granules in *B. cereus* and *B. subtilis* was conformed successfully by Sudan black B staining. The PHB granules were observed in black color and cells were pink.

3.2 PHB Production by *E. crassipes* substrate:

Two types of media were prepared for each bacterium (Table 1).

Table 1: PHB production by *B. cereus* and *B. subtilis* using Basal media and Minimal salt media in solid surface and submerge fermentation:

Media	Fermentation	Bacterium	*E. crassipes* as Carbon source	Cell dry weight µg/10 ml	PHB conc. µg/10 ml	% of PHB
Basal Media	Solid surface fermentation	*B. cereus*	Fine powder	550	147.4840758	26.8152865
		B. subtilis	Fine powder	550	168.7857865	30.68832481
	Submerge fermentation	*B. cereus*	Hydrolysate	550	90.87477958	16.5226872
		B. subtilis	Hydrolysate	550	215.2235158	39.13154833
Minimal sault Media	Solid surface fermentation	*B. cereus*	Fine powder	550	107.8096	19.60174545
		B. subtilis	Fine powder	550	23.2951	4.235472727
	Submerge fermentation	*B. cereus*	Hydrolysate	550	107.4901	19.54365455
		B. subtilis	Hydrolysate	550	212.1348	38.56996364

The higher amount of PHB production, using basal media, was found 26.81% for *B. cereus* with solid surface fermentation; and 39.13% for *B. subtilis* with submerge fermentation (Table 1).

The higher amount of PHB production, using minimal sault media, was found 19.6% for *B. cereus* with solid surface fermentation; and 38.56% for *B. subtilis* with submerge fermentation (Table 1). Regarding to these results optimization was followed by using Basal media; with solid surface fermentation for *B. cereus* and submerge fermentation for *B. subtilis*.

3.3 Incubation time optimization

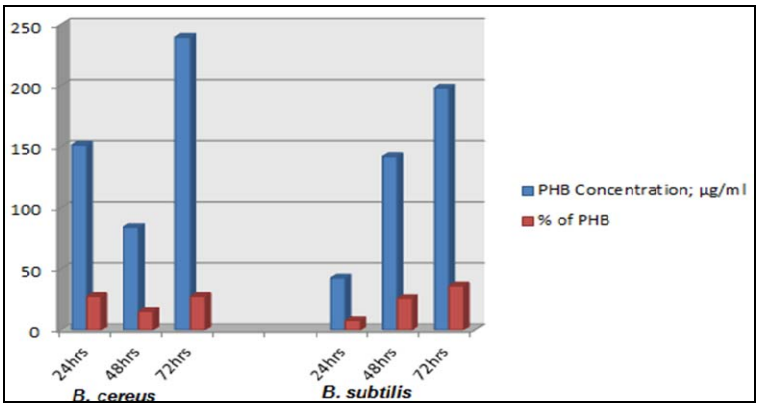

Fig 2: The effect of different incubation time on PHB Production and yield in *B. cereus* and *B. subtilis*.

The higher amount of PHB yield was found 239.613 µg/10 ml at 72 hrs incubation time for *B. cereus* and 197.915 µg/10 ml at 72 hrs incubation time for *B. subtilis* (Fig. 2). At 72 hrs incubation time microbes have more time to produce the maximum amount of PHB granules. So 72 hrs incubation time was considered as the constant and then was proceeded for pH optimization.

3.4 pH optimization

The higher amount of PHB yield was found 110.525 µg/10 ml at pH 7.2 for *B. cereus* and 197.915 µg/10 ml at pH 7.2 for *B. subtilis* (Fig. 3).

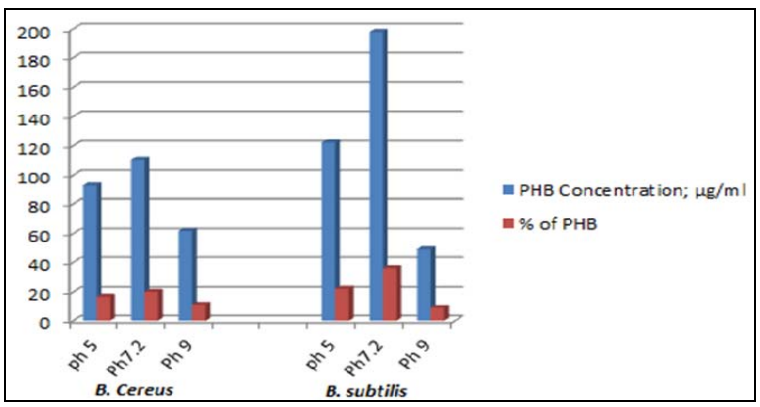

Fig 3: The effect of pH on PHB Production and yield in *B. cereus* and *B. subtilis*.

pH 7.2 is the constant optimum pH for basal media in which bacteria can produce maximum PHB granules. So pH 7.2 was considered as the constant and then was proceeded for different percentages of Nitrogen source optimization.

3.5 Different percentage of Nitrogen source optimization:
The higher amount of PHB yield was found 186.253 µg/10 ml at 1% Nitrogen source for *B. cereus* and 178.105 µg/10 ml at 0.25% Nitrogen source for *B. subtilis* (Fig. 4).

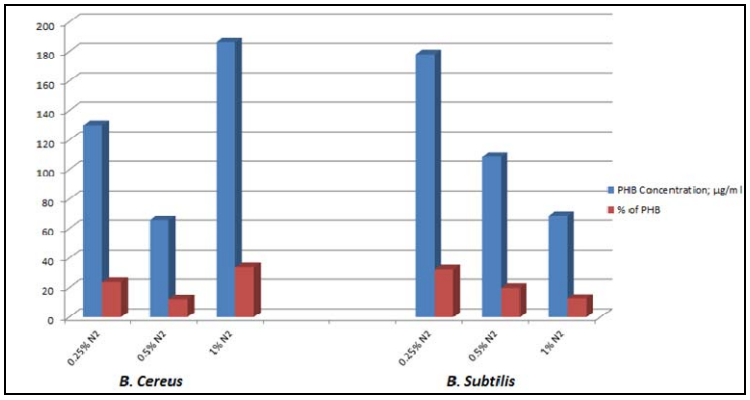

Fig 4: The effect of different nitrogen concentration on PHB Production and yield in *B. cereus* and *B. subtilis*.

4. Discussion

Screening and characterization of PHB producing bacteria from *B. cereus* and *B. subtilis* using *E. crassipes* as carbon source was studied and the result obtained is discussed below. The PHB granules were recognized by their affinity for the dye Sudan black, which is a presumptive test for presence of PHB [13]. High production costs have not enabled a full commercialization of bioplastics. So the uses of inexpensive substrate have been adopted to bring down production costs. Therefore *E. crassipes* which is freely available raw material for PHB production was used as carbon source. *E. crassipes* in elemental composition is about 12.8% nitrogen content, 36-40% carbon, 8% hydrogen and 13-14% oxygen. The standard Crotonic acid assay was done for PHB estimation. The Crotonic acid assay is very simple, easy and cost effective method for PHB estimation [14]. In this procedure the PHB react with concentrated sulfuric acid and converted into Crotonic acid. PHB production was done in Solid surface fermentation with *E. crassipes* powder and Submerge fermentation with boiled filtrates of *E. crassipes* powder by inoculation of *B. cereus and B. subtilis* to the basal media. After 48 hrs PHB crystals were obtained by following standard extraction method of PHB from inoculated production media. The present study revealed that *E. crassipes* could be used for PHB production. The extraction of PHB was done with hot chloroform treatment. In this method sodium hypochlorite was used to rupture the cell wall of bacteria to extract PHB granules [15].

Optimization of PHB production was done at different nutritional parameter such as incubation time, PH and Nitrogen concentration. PHB production by *B. cereus* optimized at 72 hrs incubation time, pH 7.2 and 1% Nitrogen source. *B. subtilis* was optimized at 72 hrs incubation time, pH 7.2 and 25% Nitrogen source; Based upon above results, we may conclude that optimum culture condition for PHB production for *B. cereus* is 72 hrs incubation time, pH 7.2 and 0.25% Nitrogen source; and optimum culture condition for PHB production for *B. subtilis* is 72 hrs incubation time, pH7.2 and 1% Nitrogen source. Concentration of PHB and percentage of PHB by *B. cereus and B. subtilis* in Solid surface fermentation with *E. crassipes* powder and Submerge fermentation with boiled filtrates of *E. crassipes* powder was calculated. The maximum amount of PHB produced by *B. cereus* in Solid surface fermentation and by *B. subtilis* in Submerge fermentation. From the results it was concluded that concentration of PHB by *B. cereus* in submerge fermentation is more efficient than solid surface fermentation; and by *B. subtilis* in Solid surface fermentation was more efficient than Submerge fermentation. Hence, from present investigation, it was concluded that *E. crassipes* is promising substrate for PHB production.

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