

Original Research Article

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A Study on Impact of Agronomical Carbon Sources in Bacterially Produced PHA

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ABSTRACT

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Addiction on conventional plastics and their illimitable usage have resulted in waste accumulation and greenhouse gas emissions. Recent technologies are rapt headed for the development of bio-green materials in order to protect our environment. A biologically-synthesized plastic, polyhydroxyalkanoate (PHA) has been a major hope due to its similar physical properties to synthetic plastics. The selections of suitable bacterial strains, inexpensive carbon sources, efficient fermentation and recovery processes are important aspects that should be taken into consideration for the commercialization of PHA. This study is intended to utilise available agronomical by-products as carbon source to accumulate more PHA by bacterial isolate KSN5 *Bacillus* sp. In this present study molasses, coir pith, coconut oil cake, paddy chaff and vermicompost were used at various concentration. In present study among the different carbon sources utilized, the bacterial isolate KSN5 *Bacillus* sp. accumulated 95% (CDW 20.54±0.14g/L) PHA in presence of 4% molasses. Next to that, the medium supplemented with coconut oil cake was found to be a substrate for growth and PHA accumulation.

Introduction

Environment protection and sustainable product development has been our target for last few years, and there has been an extensive research going on at various ways. Consequently, biodegradable plastic for example Polyhydroxyalkanoates (PHA) seems to be a significant polymer almost equal to plastics. PHA, a polyester compound, is becoming more interesting among the group of biodegradable plastics.

This polymer has a wide range of potential applications because of its desired features

such as biocompatibility, biodegradability, naturally accumulated by several bacterial species and negligible cytotoxicity to the cells. Hence, the potential application of PHA is being attained recognition in various fields involving packaging, medical and coating materials. These desirable properties in compounding and blending have broadened their applications.

One of the most important difficulties in the commercialization of PHA is the high production cost when compared to those

from the production of petrochemical-based plastic materials. From an industrial process point of view, the optimal PHA producing microbial strains should fulfil many requirements such as high accumulation rates, ability to utilize cheap carbon sources and fast growth.

Castilho *et al.*, (2009) stated that the market price of commercially available PHA (approximately 10 to 12 € per kg) is relatively greater than that of conventional synthetic plastics. The high manufacturing costs involved during the production of these polymers is mainly responsible for hampering the commercialization of biodegradable plastics. The major factors affecting the overall economics of PHA production are its content, yield and productivity, the cost of the raw materials and the recovery methods. Among these various factors, the raw material costs dominate the manufacturing costs said by Van-Thouc *et al.*, (2008). Therefore, for economical PHA production inexpensive substrates that can be used as carbon sources for bacterial strains to synthesize large quantities of intracellular PHA are necessary. By-products such as molasses, straw, bagasse generated in the agricultural sector are available abundantly and are generally used as cattle feed since they have little economic value. These agricultural residues are rich in carbohydrates and the use of such materials for the synthesis of value-added products can be advantageous and also contribute significantly to the reduction of their disposal costs (Thomsen, 2005; Castilho *et al.*, (2009).

The identification of alternative cost-effective substrates for the production of PHA has become an important objective for the commercialization in this research. By utilizing some agronomical by-products available at Thanjavur District, the present study enhanced the accumulation of PHA.

Materials and Methods

Sampling sites

In Thanjavur district, molasses, coir pith, coconut oil cake, paddy chaff and vermicompost are available more and used as cattle feed, fuel and manure in rural areas. As they contain more carbon content in different form, they were used in the present study as a crude carbon source after hydrolysis for PHA production. The afore said wastes were collected from Arignar anna sugar factory, Kurungulam, Coir factory, at Vallam, local oil mill, Indian Paddy processing unit and Sriamman Biocare, Thanjavur respectively.

Source optimization

Characterization of agronomical by products

The selected substrates were characterized for moisture content, total sugar, total reducing sugar C%, N% and pH. Total sugars were estimated using the Anthrone method (Dreywood, 1946). The amount of total reducing sugars was the key aspect for the selection of the substrates. The total reducing sugars were estimated by Dinitrosalicylic acid method (DNS) Method (Miller, 1959). The carbon and nitrogen content was determined using the CNS-analyzer (IICPT, Thanjavur). pH and the moisture content were estimated as given below.

Moisture

A clean and empty silica crucible was heated at 103 °C to 105 °C for an hour in a hot air oven and allowed to cool down to room temperature in desiccators and weighed. All the afore said carbon sources i.e substrate (10 g) was weighed accurately in the silica crucible and dried at 103 °C to

105°C to a constant weight. Moisture content was calculated. The dried samples were retained for further analysis.

pH

The pH of waste extracts were checked using Orion pH meter after calibrating the instrument with standard pH buffers.

Pretreatment and processing of the agronomical by-products

All the by-products were collected in a sterile container and washed in water then sun dried for two days. All were dried at 80°C for overnight in hot air oven. Each dried sample was ground to fine powder and passed through a 35-mesh sieve. Concentration of cellulose, hemicellulose and lignin were estimated (Updegraff, 1969; Goering and Vansoest, 1970). Each waste was first partially delignified by autoclaving (120°C, 15 lb, 20min). The mixture was kept at room temperature ($37 \pm 2^\circ\text{C}$) for 24h. They were hydrolyzed by 0.5-5% v/v sulphuric acid (solid: liquid (1:10-1:20) and autoclaved at 121°C, and 15 psi for 30 min. The hydrolyzed samples were filtered and supernatants were neutralized using sodium hydroxide (6N).

Total sugar

Anthrone solution was prepared by dissolving 0.1 g of Anthrone (9,10-dihydro 9 Ozoanthracene) in 76% sulphuric acid to a volume of 100 ml. Glucose stock standard solution was prepared in double distilled water by dissolving 10 mg of glucose in 1 ml and making a working stock of 1 mg ml⁻¹. The standard stock solution was taken in aliquots of 0.5, 1.0, 1.5, 2, 2.5, corresponding to 50, 100, 150, 200 and 250 µg of glucose respectively in test tubes and the final volume was made to 5ml by adding distilled water. A reagent blank was also

run along with the standards. All the tubes were transferred to an ice bath and 10 ml ice-cold anthrone reagent was added to each of the tubes gradually. Afterwards the tubes were kept in boiling water bath for 16 min. The tubes were then transferred to an ice bath and allowed to stand for 10 minutes at room temperature. The blue green colour developed was read for absorbance at 620 nm of wavelength using a Spectrophotometer (U-2000, Hitachi, Japan).

Reducing sugar

The DNS method was followed for estimating the concentration of reducing sugars (G. Miller, 1959). Standard solution in the range of 0 to 2 ml was pipetted out. The final volume in all the tubes was made up to 2 mL with distilled water concentrations ranging from 0 to 750 mg.1 mL DNS reagent was added to all the test tubes and mixed well. The capped test tubes were kept in a boiling water bath for 10 minutes. The tubes were taken and cool to room temperature. Extinction at 540 nm was read against the blank. Standard curves of the sugars were prepared to estimate the concentration of the unknowns.

Mass production of PHA by constructive bacterial isolate

Optimum concentration of the hydrolysate was determined by incubating 5% (V/V) of the culture in PY medium (Glucose - 20g, Peptone - 5g, Yeast extract - 3g, Agar - 15g, Distilled water- 1000ml) with 1 to 5% hydrolysates of agronomical carbon sources and incubated at room temperature for 48 to 72 hrs on shaker incubator. Then the bacterial isolate culture was inoculated in the PY medium and incubated at room temperature for 48 to 72 hrs on shaker incubator.

Extraction and quantitative assay

The spectrometric chemical assay for the determination of PHA from the sample was estimated by using Law and Slepecky, 1961 method. Suspension of the culture was centrifuged at 10,000rpm for 15 min. The pellet was re-suspended in a volume of 30% sodium hypochloride equal to the original volume of suspension at 37°C for 20 min. After 1 hour at 37°C, the lipid granules were centrifuged, washed with water and then washed with Acetone and Ethanol. The

polymer was dissolved in chloroform and kept for complete evaporation. Then 5ml of concentrated H₂SO₄ was added and heated for 40 min at 100° C in a water bath. The resultant crotonic acid was measured at 235nm against H₂SO₄ as blank in UV –VIS spectrophotometer (Beckman DU 40). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA (Zakaria *et al.*, 2010). The dried power of PHA was calculated by the formula given below.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracted PHA (g/L).

PHA accumulation (%) = $\frac{\text{Dry weight of extracted PHA (g/L)} \times 100\%}{\text{CDW (g/L)}}$

Then the PHA quantification procedure was followed as above mentioned.

Results and Discussion

Analysis of the physico-chemical parameters of the agronomical by-products

All the agronomical by-products were evaluated for their physical characteristics with respect to colour, smell, form, texture, specifically pH and moisture content might influencing the utility by bacterial isolate (Table 1). Expect molasses, all other wastes were in dry condition. The pH of all the by-products was observed almost below pH 7.0. In vermicompost, it was found as 7.0-8.2. 30.36% of moisture content was observed in vermi compost and 22.5% in molasses. 0.95%, 5.54%, 3.70% of moisture content, was observed in coir pith, rice chaff, and coconut oil cake respectively.

High moisture content was observed with molasses as compared with the other agricultural by-products. Therefore this waste needs to be dried before storage to

elude growth of unwanted microorganisms. The pH of all the by-products was below pH 7.0 proposing the acidic nature of these wastes.

Chemical composition of the agronomical by products was estimated and presented in Table 2. The amount of total reducing sugars was the key aspect for the selection of the substrates. A high percentage of this carbon is in the form of carbohydrates as seen from the total sugar content of these residues.

The presence of reducing sugar was estimated as 0.32 g/L, 0.164 g/L, in molasses and coconut oil cake respectively. The total sugar was estimated in molasses as 0.854 g/L, 0.0227 g/L in Coir pith, 0.06 in rice chaff, and 0.778 in coconut oil cake were observed. In vermicompost, there was no significant amount of reducing sugar present. All the by-products designated were rich in carbon content, i.e. 54.2% in coconut oil cake, 50.2% in rice chaff, 47% in molasses, 45.8% in coir pith and 21.7% in vermicompost were observed.

A high percentage of this carbon is in the form of carbohydrates as seen from the total sugar content of these residues. In case of molasses and coconut oil cake, significant amount of total sugars was found even though presence of more than 40% of carbon in these by-products was estimated. Coconut oil cake consists of 4.8% nitrogen content. In vermicompost, 1.84%, rice chaff, 1.4% and in molasses 0.4% of nitrogen content were observed. In coir pith there nitrogen content was not found significantly. In case of rice chaff, insignificant amount of total sugars was found even though presence of more than 40% of carbon in these by-products was estimated. The weak growth of the isolates and their inability to utilize these by-products can be documented to the presence of carbon in a form not metabolizable by the isolates.

Mass production of PHA by utilizing agronomical by-products

In present study among the different carbon sources utilized, the bacterial isolate KSN5 *Bacillus* sp. accumulated 95% (CDW 20.54±0.14g/L) PHA in presence of 4% molasses (Table 3) and 86.26 % (CDW 18.14±0.01g/L) in presence of coconut oil cake at 5%. In the medium supplemented with rice chaff, KSN5 *Bacillus* sp. accumulated 68.24% respectively. Among the different concentrations of coir hydrolysate experienced, the maximum yield was noted in medium containing 3% of the coir hydrolysate. KSN5 *Bacillus* sp. showed 38.46% (CDW 6.42±0.15g/L) and 49.89% (CDW 9.41±0.01g/L) PHA in presence of vermicompost at 3%. However, the growth of the isolates was slow in presence of rice chaff; the isolates were able to accumulate PHA upto (68.24%) by KSN5 *Bacillus* sp. followed by coconut oil cake. It may produce more based on prolonged incubation. The medium supplemented with

vermicompost produced 49.89% of PHA and more CDW based on its carbon content as they are the organic manure, enhance the growth. From the experiment, it was observed that molasses could be the best cheap waste carbon substrate. Next to that, the medium supplemented with coconut oil cake was able to serve as a substrate for growth and PHA accumulation.

Impact of the agro-industrial by-products on PHA accumulation

Among the carbon sources used molasses yielded more PHA and CDW as supported by Prabakaran and Senthilkumar, (2000). Molasses is a sucrose-rich by-product of sugar manufacturing industry as an inexpensive carbon source. It is considered to be an ideal substrate for fermentative processes. Molasses served as an outstanding source of carbon since all the isolates were able to splendidly supplement with it. The enhanced growth and PHA production of the isolates can be credited to the additional nutrients such as vitamins and minerals found in molasses which function as growth factors (Oliveira *et al.*, 2004). Gouda *et al.*, (2001) informed about the maximum PHA production by using *B. megaterium* with cane molasses and glucose as sole carbon sources 40.8 and 39.9% DCW respectively. *B.thuringensis*R1 cells were found to accumulate 22.95 and 31.36% DCW as PHA in the presence of molasses and table sugar, respectively as stated by Rohini *et al.*, (2006).

Albuquerque *et al.*, 2007 reported about strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses as the best carbon source. Maria Celisa and Nimali Prabhu, 2009 experimentally proved that molasses to be excellent carbon substrates for PHA accumulation by

Bacillus sp. In the proposed study, molasses was proved as the best carbon source to accumulate more PHA and the bacterial isolate KSN5 *Bacillus* sp. accumulated 95% with CDW 20.54± 0.14 g/L of PHA.

Recently, a few studies have been conducted using oil cakes such as sesame, mustard and palm under submerged fermentation conditions and bagasse soy cake using solid-state fermentation (Sharma *et al.*, 2007). Medium augmented with coconut oil cake was able to serve as a substrate for growth. Isolate KSN5 accumulated more PHA using this when compared with other agro-industrial residues. Thakor *et al.*, (2005) found the coconut oil as one of the best

carbon source for *Comamonas testosteroni*. As there was high carbon and little nitrogen content present in coconut flour the isolates may able to accumulate PHA. Preliminary evaluation of coconut oil cake, an agroindustrial residue recommended it to be a potential carbon source for PHA production. In our projected experiment, the isolate KSN5 *Bacillus* sp. exhibited maximal biomass (CDW 18.14±0.01g/L) and 86.26 % PHA. The exclusion of adherent fatty acids from the coconut oil cake hydrolysate was attained by a brief hexane wash thereby enabling the high quantification process (Lee *et al.*, 2000; Maria Celisa Santimano *et al.*, 2009).

Table.1 Physical characteristics of agronomical byproducts

S. No.	Samples	Colour	Wet/dry	Smell	Form	Texture	pH	Moisture Content
1	Molasses	Dark Brown	Wet	Sweet	Syrups	Viscous	5.5-5.8	22.5%
2	Coir pith	brown	dry	sweet	mass	fibrous	6.5-6.9	0.95%
3	Rice chaff	Golden brown	dry	starchy	Small shell	fibrous	6.0-6.5	5.54%
4	Coconut oil cake	Grey	dry	oil	mass	granular	6.0-6.4	3.70%
5	Vermi compost	black	dry	organic	mass	Fine granules	7.0-8.2	30.36%

Table.2 Chemical composition of selected agronomical by –products

S. No.	By-products	Reducing Sugar (g/L)	Total sugar (g/L)	Carbon (%)	Nitrogen (%)
1	Molasses	0.32	0.854	47	0.4
2	Coir pith	Nil	0.0227	45.8	Nil
3	Rice chaff	Nil	0.06	50.2	1.4
4	Coconut oil cake	0.164	0.778	54.2	4.8
5	Vermicompost	Nil	Nil	21.70	1.84

Table.3 Effect of agronomical carbon sources on bacterial isolate KSN5 *Bacillus* sp.

S. No.	Name of the Carbon source	% of carbon source									
		1%		2%		3%		4%		5%	
		CDW (g/L)	PHA (%)	CDW (g/L)	PHA (%)	CDW (g/L)	PHA (%)	CDW (g/L)	PHA (%)	CDW (g/L)	PHA (%)
1	Molasses	12.11±0.11	76.24	15.21±0.05	89.01	17.64±0.01	92.23	20.54±0.14	95.01	16.12±0.03	75.42
2	Coir pith	2.08±0.02	19.12	2.55±0.02	31.11	6.42±0.15	38.46	5.32±0.05	31.81	4.16±0.02	28.45
3	Rice chaff	5.87±0.01	42.17	8.51±0.11	51.01	10.56±0.03	60.12	11.57±0.01	68.24	9.45±0.10	59.11
4	Coconut oil cake	9.87±0.25	54.3	10.63±0.06	61.65	16.32±0.12	74.32	17.00±0.01	80.11	18.41±0.01	86.26
5	Vermicompost	5.62±0.01	30.99	5.57±0.03	35.78	9.41±0.01	49.89	8.25±0.11	41.30	3.47±0.01	29.85

Coir pith is a byproduct of the coir processing industry posing disposal problems. Lignocellulose is the most abundant constituent of coir pith and provides simple sugars on hydrolysis. The reducing sugar content in the coir industrial waste hydrolysate was 30 ± 2.4 %. The composition and properties of coir industrial waste vary depending on the maturity of the coconut, method of and disposal, period between extraction and disposal. Coir pith obtained from fully mature nuts has higher amounts of polysaccharides and fewer water soluble salts compared with younger nuts (Savithri and Khan, 1994). Murugesan and Sathesh Prabu, 2010 studied, different concentrations of coir hydrolysate on PHA production, in which the maximum yield was noted in medium containing 3% of the coir hydrolysate. In our planned study, among the different concentrations of coir hydrolysate tried, the maximum yield was noted in medium containing 3% of the coir hydrolysate. KSN5*Bacillus* sp. showed 6.42 ± 0.15 g/L CDW with 38.46 % of PHA. The isolate was capable of growing on rice chaff when individually supplied as sole

carbon sources. In present research, KSN5 *Bacillus* sp. accumulated 68.24% PHA with 11.57 ± 0.01 g/L CDW when compared with other isolates. However, the growth of the isolates was slow the isolates were able to accumulate little amount of PHA followed by coconut oilcake. It also may produce more PHA based on prolonged incubation.

The medium supplemented with Vermicompost, KSN5 *Bacillus* sp. produced 49.89% of PHA and more CDW (9.41 ± 0.01 g/L) based on its carbon content. As they are the organic manure, they may enhance the growth rate than PHA accumulation. In this present study, when compared with other carbon sources used, obtained PHA content was low in presence of vermicompost, it might be due to the absence of significant sugar content. Further, hydrolysis of these agro industrial by-products by using dilute acid not only improved the ability of the isolates to assimilate the released fermentable sugars to accumulate PHA but also avoided the interference caused by the insolubles present in the wastes during downstream processing.

This approach might be influence the accumulation of more PHA by bacterial isolates.

The potential applications of PHA in various industries and in the medical field are hopeful. However, the production cost of PHA has been a major hitch. Consequently, the ongoing commercialization activities concentrate more in isolation of potent microbial strains, creating new types of recombinant strains, tailoring various kinds of PHA, renewable carbon resources and refined recovery processes.

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