

Biosynthesis of Polyhydroxyalkanoate blends from Cassia seed: GalactoMannan and Polyhydroxybutyrate precursors

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Abstract - Polyhydroxybutyrate, one of the foremost compound among the family of PHA is a microbial polyester synthesized by bacteria as an intracellular reserve material. Basic PHB has relatively high glass transition and melting temperatures. To synthesize PHB with various co-polymers such as GalactoMannan (GM) decreases the glass and melting temperatures as well as broadens the processing window since there is improved melt stability at lower processing temperatures. The present research work focuses on the use of inexpensive substrate for PHB production and blending, copolymerization as a means to improve its flexibility and reduction in crystallinity, promoting its use as packaging films. The *Bacillus safensis* EBT1 strain was selected for PHB production under optimum conditions of 48h, 45°C and pH of 7. The extracted PHB granules were subsequently blended with a natural polymer like GalactoMannan (GM) rich in seeds of *Cassia* sp.

Keywords: Polyhydroxybutyrate, *Bacillus safensis*, Blending, Crystallinity, Galactomannan

1. INTRODUCTION

Polymer based compounds are found in every aspect of everyday life. The fast growth of the polymer industry is attributed to the unique properties of polymers including light weight, corrosion resistance, design flexibility, and ease of manufacturing. Durability is one of the advantages of polymers, but it is also their shortcoming. Petroleum based polymers are generally not biodegradable [1, 2]. In polyhydroxyalkanoate (PHA) family, poly- β -hydroxybutyrate (PHB) is the most common and well characterized member, and received much attention as a source for a novel biodegradable plastic material. Although, the studies were demonstrate that the properties of PHB such as brittleness, low extension-to-break, and lack of flexibility limit its application [3, 4]. To overcome these, production of PHA copolymers is of current research interest. The copolymer P (3HB-co-3HV), i.e. poly (3-hydroxybutyrate-co-3-hydroxyvalerate) has two major advantages over PHB: (i) the melting point, and (ii) the level of crystallinity, which are lower than that of PHB. In addition, copolymers of P (3HB-co-3HV) with higher HV fraction tend to be softer and tougher [5, 6].

On these backgrounds, there is an urgent need for the development of biodegradable materials that would not concern the use of toxic components in their manufacture and could be degraded in relatively short times to environmental friendly products. For these reasons, the development of biodegradable materials has been one of the most important challenges. There are many studies focusing on PHB modification such as the addition of modifiers, copolymerization or blending. The main aim of the work is Blending PHB with another bio-based and/or biologically degradable material allow to adjust some properties in a wide range which also favors completely compostable materials with minimal carbon-footprint, such as are those provided by the physical blend processing technology. In this sense, poly-hydroxybutyrate (PHB) is blended with another biodegradable polymer called Galactomannan. To reduce the production cost of bioplastics and by which making the pollution free, Eco Friendly environment from non - biodegradable plastic.

2. MATERIALS AND METHODS

2.1 Preparation of PHB

A PHB producing strain *Bacillus safensis* EBT1 was grown in a production medium containing glucose as the carbon source along with yeast extract: 0.2 g, peptone: 0.5 g and NaCl: 0.5 g in 100 ml distilled water. The pH of the production medium was adjusted to 7. Then it was incubated at 45°C for 48 hrs. After the incubation period, the microbial cells were collected by centrifugation at 10,000 rpm for 10 minutes. The pellet collected was washed with acetone and ethanol and re-suspended in equal volume of 4% sodium hypochlorite solution. This solution was incubated for 30 minutes at room temperature. The whole mixture was centrifuged again and the supernatant was discarded. The pellet collected was again washed with acetone and ethanol. Thus, the extracted polymer granules were stored for further use [7].

2.2 Sample collection of blends

Various natural polymers were collected and tested for their film forming abilities. The natural polymers were chosen so as to include a wide range of polysaccharides such as glucose, sucrose, galactose, mannose, xylose, maltose, etc.

Natural polymers were chosen instead of synthetic polymers because of their biocompatibility and biodegradability. Also, because of their contribution as an inexpensive substrate in reducing the high production costs of PHB. The Table 1 lists some of the waste natural polymers considered and their sources.

Table 1. Sample collection for blends

S. No	Sample	Plant Source	Area of collection
1	Cassia seeds	Cassia tora, Cassia fistula & other Cassia sp.	Drought affected regions (Pudhukottai)
2	Resin	Moringa oleifera	From residential areas
3	Sago waste	Manihot esculenta	Sago industry
4	Sorghum stalk	Sorghum bicolor	From agricultural fields
5	Tamarind seed polysaccharide	Tamarindus indica	Thangam flour mill

2.3 Preparation of cassia gum

Raw, purified cassia seeds were purchased from Dwarkesh industries. The seeds were first roasted to remove the brittle, heat sensitive husk. The seeds are ground with little stress and sieved to separate the intact endosperm from the powdered husk and germ. The endosperm known as the splits are pulverized in a mill and made into fine powder and stored.

2.4 Preparation of Cassia gum Extract

2 g and 4 g of Cassia gum was added to 100 ml of cold distilled water each in order to obtain slurries of 2% and 4% concentrations. The slurry obtained was then boiled for 20 minutes in a water bath. The bottom residue was filtered and the top clear solution obtained was then centrifuged at 5000 rpm for 20 minutes to separate all the foreign matter. This solution was made up to 100 ml and then used as a substrate for PHB-GM copolymer.

2.5 Preparation of GM-PHB blend films

1 g of cassia gum was dissolved in 100 ml of distilled water and heated for 1 h at 60°C. PEG was added to this solution in different percentages. The solutions were blended with 1% PHB-Chloroform solution in different ratios as shown in Table 2 by magnetic stirring overnight to obtain a uniform dispersion. The viscous mixture was centrifuged at 10,000

rpm for 10 mins and the clarified supernatant solution was then casted into petridishes having area of 63 cm² and 1.5 cm wall height. Petridishes were incubate in hot air oven for 8 hours at 50° C. After drying films were removed with the help of a sharp blade and kept in desiccator for 24 hrs. Films with air bubbles, cuts or imperfections were excluded from the study. Selected films were subjected for different evaluation parameters.

Table 2. Composition of GM-PHB blend films

Percentage of PEG	GM:PHB
1%	100:0
	90:10
	80:20
	70:30
	60:40
2%	50:50
	100:0
	90:10
	80:20
	70:30
60:40	
50:50	

2.6 Preparation of Copolymers of PHB with natural polymers

Instead of using glucose alone as a substrate, various natural polymers were used in conjunction with glucose as a carbon source for the microorganisms to obtain different copolymer formulations of PHB. The procedure adopted for the production of copolymers is the same as described for the preparation of PHB except for the variation of carbon sources. The various natural polymers used along with glucose and their copolymer formulations are listed in the Table 3.

Table 3. Copolymer Formulations

Substrate	Composition	Copolymer formulation
Cassia gum extract	Galactomannan (GM)	PHB-co-GM
Tamarind seed polysaccharide	Xyloglucan (XG)	PHB-co-XG
Xanthan gum	Glucomannan	PHB-co-Xanthan
Alginate	Mannuronic, Guluronic acids	PHB-co-Alginate

2.7 Effect of different C/N ratios on PHB-co-GM production

The bacterial strain was grown in 250 ml conical flasks containing 100 ml of nutrient broth with different C: N ratios viz., 2:1, 4:1, 6:1, 8:1, 10:1, 12:1 using a 1:1 proportion of glucose: cassia gum as the carbon source and yeast extract as the nitrogen source. Then the flasks were incubated on a rotary shaker (150 rpm) at 45 °C. After 48 hrs PHB yields were quantified. Based on the data the best C: N ratio was determined.

2.8 Preparation of copolymer films

Films were prepared by dissolving 1g of the copolymer in 100 ml of distilled water containing 2 ml of Polyethylene glycol as a compatibilizer, with simultaneous heating to obtain a viscous mixture which was then casted into preheated Petri plates and dried in ovens. The films were then evaluated using various tests and they were compared to determine the best substrate.

2.9 Moisture absorption studies

Moisture Absorption was measured according to the method of Angles and Dufresne [8]. Both the blend and the copolymer films of 2 cm x 2 cm were conditioned for 24 h for the study. After that the films were weighed and were kept in a desiccator containing CaCl₂ saturated solution to obtain a relative humidity of 55%. The samples were weighed at desired intervals until they reached an equilibrium state. The Moisture Absorption of the blend films was calculated as follows:

$$MA\% = \frac{W_w - W_d}{W_d} \times 100$$

Where MA% is Moisture Absorption (%), W_d and W_w are the weights of the sample film before and after conditioning, respectively.

3. RESULTS AND DISCUSSIONS

3.1 Evaluation of PHB-Cassia blend films

Appearance of films as evaluated by visual observation was found to be opaque, yellowish films with uniform texture. An image of the copolymer film PHB-co-GM has a better visual appeal than the blend film obtained as shown in FIG 1.



FIG.1. Image of PHB-co-GM film

The thickness of the films as measured using a screw gauge at three different points was found to be in the range of 0.2 to 0.5 mm. The films with 90% and 80% GM showed better smooth surface than the blend films containing 70%, 60% and 50% GM. After the blending considerable phase separation was seen in films containing GM and PHB in the ratio 50:50. And also those blends when casted were very brittle and could not be taken as films from the Petri plate. The addition of compatibilizer that is PEG allowed to form homogenous blend. However, films containing 2% of PEG showed smooth surface and good flexibility, but the films with 1 % PEG did not possess satisfactory film properties.

3.2 Effect of substrates on the growth and production of polymers

A comparative analysis of the growth pattern of the microorganism utilizing three different substrates: glucose, a natural polymer and a combination of both is depicted in the FIG 2.

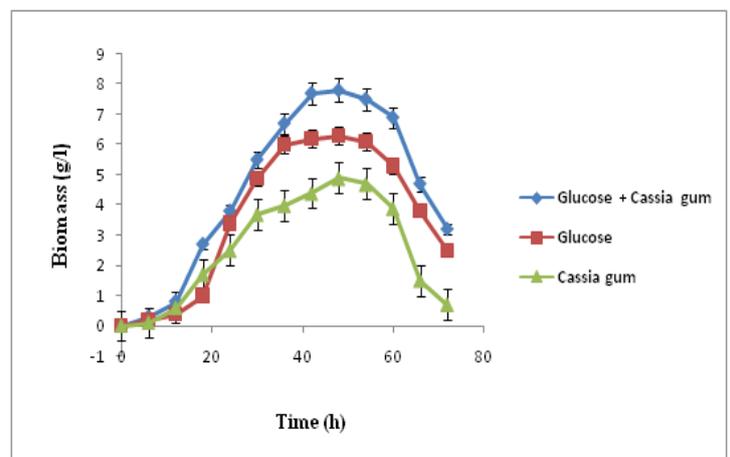


FIG.2. Effect of substrates (2% w/v) on growth vs time

This study was carried out to analyze the influence and efficacy of a natural polymer as a substrate on PHB production. From the graph it can be seen that higher growth and biomass yield (7.8 g/l) of the parent strain were observed for the combined substrates (Glucose+cassia) at optimum pH of 7, temperature of 45° C and at 48 hrs. This proves that addition of cassia gum as a feedstock along with glucose increases the yield as well as the biomass. The FIG 3. depicts the maximum biomass yield and polymer accumulation for different substrates.

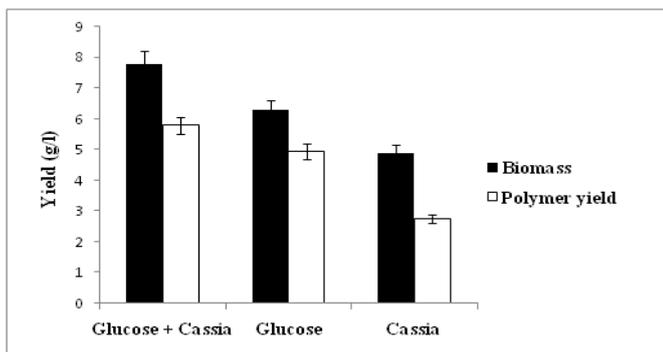


FIG.3. Effect of substrates (2% w/v) on biomass and polymer yield

The polymer production by a particular strain is also related to its biomass. Thus, high yields of the microbial polymer were observed for the glucose+cassia substrate in correspondence with the higher biomass yield. Thus cassia gum’s efficacy as both as an inexpensive substrate as well as its contribution in product formation as a copolymer is evident from both the graphs.

3.3 Effect of different C: N ratios on Copolymer yield

Under normal conditions, bacteria synthesize their body materials like proteins and metabolites. But, during limiting conditions of nutrient, bacteria may shift their protein synthesis to polymer synthesis for their survival. To exploit this phenomena, experiments were carried out to study the accumulation of copolymer with nitrogen limiting conditions. The PHB-GM yield was highest at 8:1 ratio (9.32 g/l).

As the carbon content was increased in the media keeping N as constant, up to certain limit (i.e., 8:1) polymer accumulation was increased and there off it showed a decline. This was probably due to the substrate inhibition and cell proliferation.

3.4 Moisture absorption of blend films

Water resistant is an important criterion in materials intended for packaging. The Moisture Absorption of GM-PHB blends were shown in FIG 4.

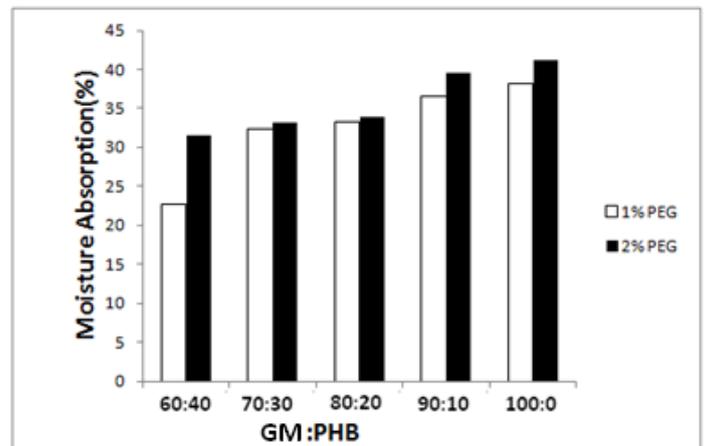


FIG.4. Moisture absorption of blend films at various compositions

The Figure clearly shows that the moisture uptake increases with decrease in PHB ratio. This indicates that blending of PHB on GM increases its hydrophobicity. The reason could be that the GM is able to form hydrogen bonds with the hydroxyl group of PHB and this strong structure could reduce the dispersion of water molecules in the material. This may also be due to the high crystalline nature of the PHB since hydrophobicity is directly related to crystalline nature. Thus, these results indicate that the addition of PHB improves the water resistance of the GM matrix. Similar results were found to be higher water retardation by polymer blends and biocomposites.

3.5 Evaluation of Copolymer films

The copolymer films produced using different combinations of carbon substrates were evaluated for their Physico-Mechanical properties represented in the Table 4.

Table 4. Physico-mechanical properties of films

S. No	Copolymer formulations	Appearance	Thickness (mm)	Folding endurance	Tensile Strength (MPa)	Water Uptake (%)
1	Extracted PHB	Yellowish White, rough	0.23	3	0.9	1.25
2	PHB-co-GM	whitish, flexible & smooth	0.366	27	4.35	7.1
3	PHB-co-XG	Brownish, Flexible	0.59	18	3.55	20.3

		and rough				
4	PHB-co-Alginate	Brownish white, coarse surface	0.408	10	3.1	47.5
5	PHB-co-Xanthan gum	whitish, smooth and transparent	0.574	6	1.8	33.8

The appearance of the films was mostly white or brown depending on the color of the natural polymer used as the substrate. The moisture uptake was found to lower for PHB-co-GM films because of the fact that GM is sparingly soluble in hot water and PHB is insoluble in water. While the other natural polymers are highly soluble in water and thus have good water uptake which is not desirable for a bioplastic copolymer. Besides PHB-co-GM shows good folding endurance and tensile strength, thus shows improved properties compared to PHB alone.

4. CONCLUSION

The present study focused on the search and use of an inexpensive carbon substrate for PHB production since substrate cost is the one of the major prohibiting factors. On lines of which Sorghum bicolor stalks, an agricultural waste was used for PHB production by *Bacillus safensis* EBT1. The yield was considerably higher with around 1.2 g/100 ml at optimum conditions of pH 7, 45°C and an incubation period of 48 hrs. Moreover, various other natural polymers were selected and tested for their film forming abilities. Cassia seeds, a natural inexpensive source of the polysaccharide Galactomannan was finally chosen for blending in view of its excellent viscosifying abilities, non-toxic nature. Thus, PHB-co-GM copolymer shows improved properties and has a good scope for industrial processability in packaging applications.

REFERENCES

[1] A.S.M. Annuar, A.M. Gumel, Y. Chisti, "Recent advances in the production, recovery and applications of polyhydroxyalkanoates". *J. Polym Environ.* 2013; 21:580-605.

[2] M. Arrieta, L. Juan, H. Alberto, R. Emilio, "Ternary PLA-PHB-Limonene blends intended for biodegradable food packaging applications". *European Polym J.* 2014; 50: 255-270.

[3] V. Gorenflo, G. Schmack, R. Vogel, A. Steinbuchel, "Development of a process for the biotechnological large-scale production of 4-Hydroxyvalerate-containing polyesters and characterization of their

physical and mechanical properties". *Biomacromolecules.* 2001; 2: 45-57

[4] Y. Chisti and E. Grothe, "Poly (β hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: Behaviour of fed-batch cultures". *Bioprocess Eng.* 2000; 22: 441- 49.

[5] R. Bhati and N. Mallick, "Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production by the diazotrophic cyanobacterium *Nostoc muscorum* Agardh: Process optimization and polymer characterization". *Algal Research.* 2015; 7: 78-85.

[6] M. Avella, E. Martuscelli, M. Raimo, "Properties of blends and composites based on poly (3-hydroxy) butyrate (PHB) and poly (3-hydroxybutyrate-hydroxyvalerate) (PHBV) copolymers". *J Mat Sci.* 2000; 35: 523-545.

[7] H.L. John and A.S. Ralph, "Assay of polyhydroxybutyric acid". *J Bacteriol.* 1961; 82: 33-36.

[8] A.S. Angles and A. Dufrense, "Plasticized starch/tunicin whiskers nanocomposites Structural analysis". *Macromolecules.* 2000; 33: 8344-8353.