PRODUCTION OF PHB FROM BACTERIA ISOLATED FROM COCONUT RETTING WATER AND EVALUATION OF THE YIELD USING DIFFERENT NUTRITIVE SOURCES

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Abstract

Poly Hydroxy Butyrate (PHB) is a biodegradable alternative to fossil fuel-based plastics, which is produced by bacteria during their stationary growth phase. Its unique physical and chemical properties make it highly useful. Because of the harmful effects of non-biodegradable plastics becoming evident, researchers now focuses on developing biodegradable plastics from cost-effective raw materials. In Kanyakumari district, coconut is the major money crop apart from rubber. The mesocorp are being used for the production of fibre. Before taking fibre from the mesocrop, it has to be processed by soaking the mesocorp in water for more than 6 months. During the process of degradation there are so many microorganisms involving. They degrade some part of the mesocorp and make fibre separable and durable. During this processing, there are lot of lignine, lanine pectine and different obnoxious gases are released into the let out retting water coming out from the retting ground and on mixing with water bodies of the back-water that spoil the total aquatic environment. In this study, bacteria present in the retting water were isolated from the retting water and the same water was utilized as the culture media to grow their isolated bacteria which produces bio plastics (PHB). In order to maximize the growth of the bacteria and production of PHB, different carbon sources such as, lactose and sucrose were added to the retting water. The phosphate and nitrate were supplied to the retting water in the form of potassium nitrate, yeast extract and dihydrogen ortho phosphate. Four bacteria such as Streptococcus thermophilus, Alteromonas sp., Vibrio parahaemolyticus and Alkaligenus eutrophus were isolated from the retting water of the retting ground. The nutritive sources such as carbon, nitrogen and phosphate were needed for the maximum yield of PHB. The PHB was quantified (OD) and assessed under different parameters. This study opens a new avenue for the utilization of coconut retting water which is found to be one of the major water pollutants in the coastal areas of Kanyakumari District and also made a way for the production of PHB from the bacteria present in the retting water.

Keywords: *Polyhydroxy butyrate (PHB), coconut retting water, PHB producing bacteria,* biodegradable plastics.

Introduction

Poly Hydroxy Butyrate (PHB) is water insoluble and relatively resistant to hydrolytic degradation. This differentiates PHB from most other current available bioplastics, which are either water soluble or moisture sensitive. PHB shows good oxygen permeability. It has good ultra-violet resistance but has poor resistance to acids and bases. PHB is soluble in chloroform and other chlorinated hydrocarbons. It is biocompatible and hence suitable for medical applications.

Now a days, researchers pave the way for the invention bio degradable plastics. Coir retting waste water is a highly toxic that poses a high risk to natural water bodies. However, the production of PHB is often limited by the high cost of essential nutrients required for bacterial growth. An innovative solution to this challenge is the utilization of bacteria capable of surviving in coir retting wastewater as a growth medium. The bacteria that can tolerate the retting water are very important to produce PHB from retting water. So, the bacteria have to be isolated from the coconut retting water it self.

Materials and Methods

The retting water sample was collected from the retting ground. The collected retting water samples were serially diluted and planed on the petriplates filled with nutrient agar culture media and is incubated for 24 hours at 37C. After incubation, different bacterial colonies were isolated and screened for the production of PHB. The colonies that produce PHB were identified by the Sudan black staining of smeared bacterial slide decolorized with Xylene and again treated with safranine. Observation of inclusion bodies is the confirmation of PHB production. The colonies that produced PHB were selected, sub-cultured and preserved for further study. The quantification of PHB was done by UV Spectrophotometer at 450 nm.

Result

Different PHB yielding bacteria were isolated from the coconut retting water (Table 1) were identified by biochemical test as follows

Table 1: Shows the result of different tests conducted to identify the isolated bacteria from the retting water

Sl.No	Name of the Organisms	Gram Staining	Motility	Ceasin hydrolysis	Gelatin hydrolysis	Citrate hydrolysis	Methyl red	Indole	Starch	Catalyse	Nitrate reduction	Vogas	Lactose	Sucrose	Urea hydrolysis
1	*Streptococcus thermophilus*	Cocci +	-	+	+	-	-	-	+	+	+	+	-	-	+
2	*Alteromonas sp.*	Cocci -	+	+	+	+	+	-	+	+	+	+	+	-	+
3	*Vibrio parahaemolyticus*	Rod -	+	-	-	+	-	-	+	+	+	+	-	-	+
4	*Alkaligenus eutrophus*	Rod +	-	+	+	-	-	-	+	+	+	+	+	-	+

Sudan Black Staining of PHB Producing Bacteria

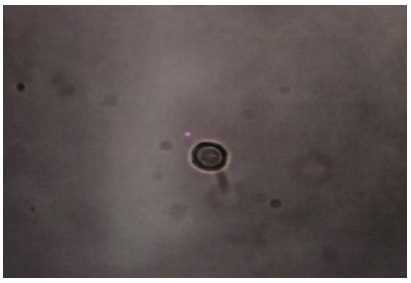

Figure 1 Sudan Black Staining of PHB Producing bacteria *Alkaligenus Eutrophus*

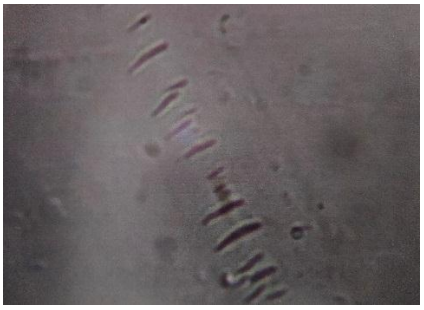

Figure 2 Sudan Black Staining of PHB producing bacteria *Vibrio parahaemolyticus*

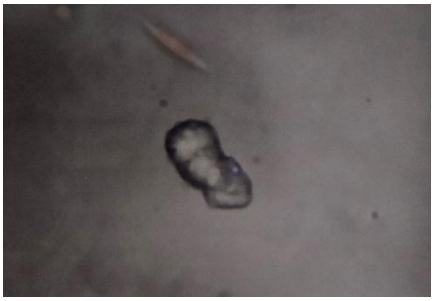

Figure 3 Sudan Black Staining of PHB producing bacteria *Streptococcus thermophilus*

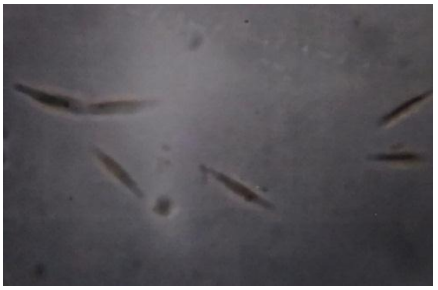

Figure 4 Sudan Black Staining of PHB producing bacteria *Alteromonas sp.*

Figure 5 Shows the Petriplates containing PHB harvested from different bacteria

Carbon Sources

Different PHB yielding bacteria were isolated from the coconut retting water and tested their yielding efficiency, when supplemented different carbon sources such as sucrose and lactose.

The isolates were when grown in culture media added with 1g of lactose, the maximum PHB yield was (0.0792) observed in *Streptococcus thermophilus* and followed by *Alkaligenus eutrophus* (0.0492), *Alteromoas sp.* (0.0151) and *Vibrio paraheamolyticus* (0.0122) (Table 2).

Table 2: Role of Lactose on the production of PHB in four selected bacteria

Sl. No	Name of the Organisms	Lactose Concentration (g)	Yield of PHB (OD)
1	*Streptococcus thermophilus*	1	0.0792
2	*Alteromonas sp.*	1	0.0151
3	*Vibrio parahaemolyticus*	1	0.0122
4	*Alkaligenus eutrophus*	1	0.0492

The isolates were when grown in culture media added with 1g of sucrose, the maximum PHB yield (1.7247) was observed in *Vibrio paraheamolyticus* and followed by the other bacteria *Alteromonas sp.* (1.6634), *Alkaligenus*

eutrophus (1.5190) and *Streptococcus thermophiles* (1.4996) (Table 3).

Table 3 Role of Sucrose on the Production of PHB in four Selected Bacteria

Sl. No	Name of the Organisms	Sucrose Concentration (g)	Yield of PHB (OD)
1	*Streptococcus thermophilus*	1	1.4996
2	*Alteromonas sp.*	1	1.6634
3	*Vibrio parahaemolyticus*	1	1.7247
4	*Alkaligenus eutrophus*	1	1.5190

Nitrogen Sources

The PHB producing bacteria isolated from the retting water collected from Rajakkamangalam retting ground were tested for the production of PHB when supplementing the different nitrogen sources in the form of potassium nitrate and yeast extract.

Vibrio paraheamolyticus isolated from the retting water yielded the maximum PHB (0.8260). The other strains such as *Alkaligenus eutrophus, Alteromonas sp*. and *Streptococcus thermaphilus* produced 0.1139, 0.0729 and 0.0560 respectively (Table 4). Among the four bacteria tested for PHB, *Streptococcus thermophilus* produced less quantity than the other three bacteria.

Table 4 Role of Potassium nitrate on the production of PHB in four selected bacteria

Sl. No	Name of the Organisms	Potassium Nitrate Concentration (g)	Yield of PHB (OD)
1	*Streptococcus thermophilus*	1	0.0560
2	*Alteromonas sp.*	1	0.0726
3	*Vibrio parahaemolyticus*	1	0.826
4	*Alkaligenus eutrophus*	1	0.1139

When the other nitrogen source, Yeast extract was when added to culture media, the *Vibrio paraheamolyticus* yielded the maximum PHB of 0.2680 (Table 5). The other bacteria such as *Streptococcus thermophilus, Alkaligenus eutrophus* and *Alteromonas sp.* produced 0.0974, 0.0491 and 0.0477 PHB respectively (Table 5). Among the four bacteria tested for PHB, the *Alteromonas sp.* produced less quantity than the other three bacteria.

Table 5 Role of Yeast extract on the production of PHB in four selected bacteria

Sl. No	Name of the Organisms	Yeast extract Concentration (g)	Yield of PHB (OD)
1	*Streptococcus thermophilus*	1	0.0974
2	*Alteromonas sp.*	1	0.0477
3	*Vibrio parahaemolyticus*	1	0.268
4	*Alkaligenus eutrophus*	1	0.0491

Phosphate Sources

In order to study the role of phosphate in the synthesis of PHB in bacteria isolated from coconut retting water, the isolated strains were cultured in media supplemented with 1g of Dihydrogen ortho phosphate, Dipotassium hydrogen phosphate and Disodium hydrogen ortho phosphate. Among these phosphate sources, the bacteria supplemented with Dihydrogen - ortho phosphate only supported the growth and hence the Dihydrogen - ortho phosphate was selected for the production of PHB on different bacteria isolated.

Among the bacteria tested with 1g of phosphate in the form of Dihydrogen ortho phosphate, *Alkaligenus eutrophus* yielded the maximum PHB (0.0792) (Table 6), and the minimum production (0.0544) was noticed *in Alteromonas Sp.*The other two isolates *Virbio paraheamolyticus* and *Streptococcus thermophilus* yielded the PHB of 0.0755 and 0.0715 respectively, which is in between *Alkaligenus eutrophus* and *Alteromonas sp.* (Table 6).

Table 6 Role of Dihydrogen Orthophosphate on the Production of PHB in Four Selected Bacteria

Sl. No	Name of the Organisms	Dihydrogen Orthophosphate Concentration (g)	Yield of PHB (OD)
1	*Streptococcus thermophilus*	1	0.0715
2	*Alteromonas sp.*	1	0.0544
3	*Vibrio parahaemolyticus*	1	0.0755
4	*Alkaligenus eutrophus*	1	0.0792

Discussion

The bacteria growing in retting water were isolated and used for this study, because the retting water is an adverse environment where the adopted bacteria can only live in this condition. There were twelve bacteria isolated from the retting water and among the twelve bacteria, four bacteria produced the PHB. They were *Streptococcus thermophilus, Alteromonas sp., Vibrio para haemolyticus* and *Alkaligenus extrophus*. From the present study it has been made known that each bacteria requires different nutritional sources for the maximum production of PHB. The same result has been arrived in other studies conducted in different laboratories. Different amounts of PHB were produced by different strains studied; however, PHB levels were 0.93 to 21.15% in MRS/M17 medium. In general, the amount of PHB produced by some *lactococcus* species was higher than that produced by *Lactobacillus* and *Streptococcus* strains. In contrast, Aslim et al. mentioned that the amount of PHB produced by some *Lactobacillus* species was higher than that produced by *Lactococcus, Pediococcus* and *Streptococcus strains.*

In this study bacteria present in the retting water were isolated and the retting water itself was utilized as the culture media to grow the isolated bacteria which produces bio plastics (PHB). In order to maximize the growth of the bacteria and production of PHB, the retting water was added with different carbon sources such as, lactose and sucrose. The phosphate and nitrogen were also supplied to the retting water in the form of potassium nitrate, yeast extract and dihydrogen ortho phosphate.

The same bacteria then supplied with different carbon sources they responded differently in the yield of PHB. Among the different carbon sources supplemented to the retting water, sucrose yielded the maximum PHB in all the four bacteria and among the tested bacteria *Vibrio parahaemolyticus* yielded the maximum PHB. From the study it has been concluded that each bacterium yielded differently in utilizing the same carbon source sucrose and therefore sucrose can be recommended for producing PHB from the retting water. The same report was proved by the study conducted the Celik et al., 2005 and in their study, they tested PHB producing bacterial species with different carbon and nitrogen sources and found that the carbon influence on PHB production. Among the two carbon source studied, sucrose yielded the maximum PHB of 32.56 w/v and followed by lactose and glucose having 29.7 and 12.47w/v PHB respectively.

The same inference has been reflected in one of the studies conducted by Aslim , *et al*., in 1998, it reported that the production of PHB by some lactic acid bacteria was found to be 35.80%(w/v) (for Lb. bulgaricus C8) for dry cell weight. The differences above, were resulted from different types of strains, types of medium and cultivation method used in individual study. Koyama and Doi (1995) reported that *Ralstonia eutropha* growing on fructose and pentanote acid in a chemostat yielded PHP (3HB-co-3HV) with molecular mass that increased along with the dilution rate of the cultures. The maximum biomass and biodegradable polymers were produced when fructose was used as the carbon source. Anderson and Dawes (1990) and Braunegg et al, (1978) showed accumulation of PHB by Alcaligenes faecalis using fructose as the Carbon source.

The nitrogen is one of the major nutritional source for the growth of bacteria, nitrogen is the main compound in amino acids and different hormones. In this experiment, two forms of nitrogen such as potassium nitrate and yeast extract were supplied in the culture medium of PHB producing bacteria and tested their role is the production of PHB. From this study it has been found that both nitrogen sources influenced almost equally for the production of PHB, but the production quantity varied in different bacteria when same nitrogen sources was given on different bacteria. Every bacterium has its own protein and

lipid profile which varies from species to species, this may be the reason that the same nitrogen source of culture media yielded different quantity of PHB. According Xi *et al.,* in 2000, the PHB production varies with nitrogen sources. They have reported that *Pseudomonas cipacia* species when examined for the production of PHB ,the amount of PHB produced in M17 broth medium was lower than the amount of PHB production in nutrient broth medium. They also determined that the PHB synthesis is highly depended with nitrogen medium only. These observation also corroborate our result. To maintain the protein lipid composition in the cell, every bacteria in take different rate of nitrogen and hence the need for nitrogen also varied based on the species.

Anderson and Dawes (1990) and Braunegg et al. (1978) showed the accumulation of PHB by *Alcaligenes faecalis* using yeast extract as the nitrogen source. Use of Yeast as the nitrogen source has been reported by Fukui (1976), Nishimura et al. (1978) and Fernandez-Castillo et al. (1986)

The culture medium of the bacteria was also supplemented with phosphate in the form of dihydrogen ortho phosphate. The phosphate are the major compound in nucleic acid. The result obtained from the study evidence that the same quantity of phosphate when supplied to different bacteria isolated from retting water produced different quantity of PHB and hence it has been concluded that apart from the effect of phosphate sources and quantity, the type of microorganism or different type of microorganism produces the PHB differently utilizing the same quantity of phosphate.

Conclusion

The present study was carried out to find out the production of PHB yielding bacteria from Coconut retting water and to optimize the chemical and nutritive parameters for the maximum yield of PHB. Four bacteria, *Streptococcus thermophilus, Alteromonas sp., Vibrio parahaemolyticus* and *Alkaligenus eutrophus* were isolated from the coconut retting water produced PHB. They yielded of PHB in relation to their species and type of nutritional sources. The important new finding in this study is the utilization of the harmful pollutant, coconut retting water, as a source of culture media which help to reduce the pollution of brackish water in Kanyakumari district and also earn money from the wasting retting water

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