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Maximization of poly-β-hydroxy butyrate production by rhizo-bacteria using eco-friendly agri-byproducts

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Abstract

Attempts have been made, using rhizobacteria, to develop a protocol for economical polyhydroxybutyrate production using agri-byproducts. The optimum conditions for maximum PHB production were found with modified glucose minimal media. A cost effective media using exclusively readily available agri-byproducts yielding as much as 60 mg/l PHB (which correspond to 53.1 % of the biomass) from isolates of rhizobacteria has been developed

Keywords *Polyhydroxybutyrate**,** Agri-byproducts, Rhizosphere bacteria, Optimization ·Biodegradable plastic*

1. Introduction

Polyhydroxyalkanoates (PHAs) are known to be accumulated as intracellular inclusion in some bacteria as carbon and energy reserves when surplus carbon is available, under the condition of limiting nutrients like nitrogen, sulphur and phosphorus. Beside nitrogen which play an important role in PHA synthesis, levels of phosphorus and sulphur also play a significant role[1]. A large number of bacteria including *Alcaligens, Bacillus, Pseudomonas, Azotobacter* and recombinant *E.coli* have been found producing good amount of PHAs. There has been considerable interest in the development and production of biodegradable polymer to solve the current problem of pollution caused by the continuous use of synthetic polymer of petroleum origin based synthetic plastics (Lee, 1996). The possibility of developing an economical protocol for commercial scale is very expensive which restricts for other important applications. Most of the chemical and physical properties are similar to conventional plastic and biodegradability is one important property which separates them from conventional plastics. Because of their high cost of production, efforts are being made to use low cost agri-byproducts like cotton cake, mustard cake, molasses and potato peel as a supplement of expansive nitrogen and carbon sources in media to reduce its high cost. It was reported that microorganisms can utilize the potato processing waste (Rusebdu and Sheppard, 1995) and biowaste (Jacobus, 2001) as carbon source for production of polyhydroxyalkanoates although generally it is believed that *Rhizobium* can not utilize starch but some *Rhizobium* strains can utilize potato waste which are good source of starch (de Oliveirs, 2007). The objective of the present study was to maximize production of polyhydroxybutyrate by rhizobacteria using agri-byproducts and/or household waste as carbon and nitrogen sources for the development of an economical protocol.

2. Materials and Methods

Rhizobial strains were isolated from root nodules of leguminous plants (Chickpea and Barseem) and other rhizobacterial strains were isolated on YEMA medium from oil spilled soil and soiled sewage water. Out of a total of 150 isolates based on orange/yellowish florescence produced by these strains under UV light using Nile blue dye (Ostle and Holt, 1982), two rhizobial strains B4, C150 and a rhizobacterial strain P17 were selected for present study. These isolates were evaluated for PHB production on the basis of growth on glucose minimal media and for presence of fluorescence. The screening for intensity of fluorescence indicates the quantity of PHB produced. The biomass were harvested by centrifuging them at 6500 rpm and bacterial cells were lysed with sodium hypochlorite hydrolysis (Law and Slepecky, 1961) and chloroform and PHB extracted using chloroform. An aliquot each was heated at 65 °C for 48 h and dried till a constant weight was obtained to compute the cell biomass. The PHB yields was determined and computed and expressed as micro grams per unit mass of cells.

3. Results and Discussion

To make the PHB production a cost effective process and to develop an economical protocol for PHB production, formulations of media containing carbon and/or nitrogen from agri-byproducts were utilized as supplement for expansive carbon and nitrogen source. Various media modifications carried out in basal media are given in table 1.

Maximum quantity of PHB yields found were 19.5 to 67.6 % with glucose as carbon source with KNO_3 (0.3 g l^{-1}) and Yeast Extract (0.3 g l^{-1}) as nitrogen source. Keeping this as control media several component substitutions were attempted to optimize PHB production. The optimum condition for maximum PHB productions were found when modified glucose minimal media were used having the following compositions: molasses (2 g l_{-1}), KNO_3 (0.3 gl_{-1}), yeast extract (0.3 gl_{-1}), K_2HPO_2 (0.6 gl_{-1}), $MgSO_4.7.H_2O$ (0.05 gl_{-1}) and $CaCl_2.$ $2H_2O$ (0.02 gl_{-1}) and another media with potato peels (4 gl_{-1}), yeast extract (0.6 gl_{-1}), K_2HPO_2 (0.6 gl_{-1}), $MgSO_4.7.H_2O$ (0.05 gl_{-1}) and $CaCl_2.2.H_2O$ (0.02 gl_{-1}).

3.1 Replacing Carbon Source with Molasses

Molasses were used as carbon source supplementing the glucose in the media and the PHB production ranged from 9.6 to 70.1 %. The maximum PHB percentage was 70.1 % obtained from bacterial strain B4 where 61.0 mg/l of PHB could be recovered from the bacterial cell's fresh weight of 517 mg/l and biomass of 87 mg/l. When amount of molasses kept constant and 0.5 yeast extract was replaced with mixture of 0.3 g KNO_3 and 0.3 g yeast extract the percentage of PHB yield was almost double from bacterial strain P17. It means mixture of nitrogen can work as ideal substrate for PHB production. In other treatments no considerable change was observed. Strains of *Rhizobium japonicum species* (*Cicer*), *Bradyrhizobium japonicum* USDA 110 and *Rhizobium* species 2426 produced 0.285 g/l PHB[8]. In an another study on *Alcaligenes eutrophus* growing on ammonical nitrogen and cane molasses[9] yielded up to 26.5 % PHB of dry cell weight.

3.2 Replacing Carbon Source with Potato Peels

PHB production were 27.9 to 63.8 % percent when potato peels were used as principal carbon source. The maximum PHB percentage was 63.8 % in case of bacterial strain C150 with PHB yield was 50 mg/l while bacterial cell's fresh weight was 445 mg/l and biomass 78.4 mg/l. PHB production was nearly double using potato peels as carbon source and 0.6 g yeast extract in bacterial strain P17. This suggests that potato peels and yeast extract act as good carbon and nitrogen source for bacterial strain P17. The PHB percentage as high as over 90 % of dry cell weight have been reported in case of *Methylobacterium* using glucose and lactose as carbon source (*Ghatnekar et al.,* 2002).

3.3 Replacing Nitrogen Source with Cotton Cake

When cotton cake was used as main nitrogen source to replace KNO_3 in minimal media the PHB production were 13.7 to 65.9 % (table 1). Maximum PHB percentage was 65.9 % with PHB yielding 29.0 mg/l while bacterial cells fresh weight was 300 mg/l and biomass was 44 mg/l in case of bacterial strain B4. When amount of cotton cake was kept constant and instead of mixture of 0.075 g yeast extract and 0.075g KNO_3 only 1.2 g yeast extract used PHB percentage increased from 31.1 to 65.8 percent. No important change were observed in all other combinations.

3.4 Replacing Nitrogen Source with Mustard cake

When mustard cake was used as principal nitrogen source the PHB production ranged from 8.9 to 61.8 percent. The maximum PHB percentage was 61.8 % in case of bacterial strain B4 along with 40.2 mg/l of PHB while the bacterial cell's fresh weight was 440 mg/l and biomass was 65 mg/l. Considerable changes in percentage of PHB were observed when amount of mustard cake was reduced to 1.2 g from 2.4 g/l media. The PHB percentage increased from 15.1 to 61.8 percent in case of rhizobial strain B4 while rest of the bacterial strains showed no important change in PHB production

Beside the above formulations media containing exclusively agri-byproducts were also used to make a cost effective formulations for efficient PHB production. The various media modifications were carried out to increase PHB percentages are listed in table 2. Bacterial strain B4 when grown on a media containing potato peel (6 g l_{-1}) with mustard cake (3 g l_{-1}) produced PHB yields of the order of 50 % dry cell weight (table 2) and another media with same bacterial strain B4 have PHB accumulation up to (53.1% dry cell weight) when media containing molasses (3 g l_{-1}) and mustard cake (3 g l_{-1}) are incorporated in the media

Table 1. Percent PHB production on diverse carbon and nitrogen sources incorporated into the growth media.

S.No.	Carbon source	Nitrogen source	B4	P17	C150
1	Glucose 2 g	0.3 g Y.E. + 0.3 g KNO_3	21.9	67.6	19.5
Replacing Carbon Source with Molasses					
2	Molasses 2 g	0.5 g Y.E.	69.6	59.8	22.4
3	Molasses 2 g	0.3 g KNO_3 + 0.3 g YE	70.1	59.8	49.6
4	Molasses 4 g	0.5 g Y.E	31.6	28.5	9.6
Replacing Carbon Source with Potato peels					
5	Potato peals 2 g	0.6 g KNO_3	27.9	44.7	29.5
6	Potato peels 4 g	0.6 g Y.E.	41.1	40.8	63.8
Replacing Nitrogen source with Cotton cake					
7	Glucose 2 g	0.075 g Y.E. + 0.075g KNO_3 + 1.2 g Cotton cake	37.5	41.6	30.1
8	Glucose 2 g	0.15 g KNO_3 + 1.2 g Cotton cake	65.9	13.7	48.8
9	Glucose 2 g	0.075 g Y.E. + 0.075 g KNO_3 + 2.4 g Cotton cake	34.8	28.7	31.1
10	Glucose 2 g	0.12 g Y.E. + 2.4 g Cotton cake	33.3	15.1	65.8
Replacing Nitrogen Source with Mustard cake					
11	Glucose 2 g	0.12 g Y.E. + 2.4 g Mustard cake	15.1	50	36.1
12	Glucose 2 g	0.15 g KNO_3 + 1.2 g Mustard cake	61.8	42.2	8.9
13	Glucose 2 g	0.6 g Y.E. + 1.2 g Mustard cake	24	24.3	19.1

Table 2. Efficiency of PHB production on exclusively agri-byproducts**.**

S.No.	Strain	Carbon Source	Nitrogen Source	PHB (mg/l)	PHB %
1	P17	Glucose 2 g ©	0.3 g yeast extract + 0.3 g KNO_3	22	67.6
2	B4	Potato peel 6 g	Mustard cake 3 g	43	50
3	B4	Molasses 3 g	Mustard cake 3 g	60	53.1

4. Conclusions

PHB percentage up to 53.1 can be obtained by utilizing media containing exclusively agri-byproducts. Beside this certain rhizobial species are also found which can easily utilize the starch which is generally not the normal trend. In the present study, some rhizobial strains were also isolated which are able to utilize the starch present in potato peel. Optimum C: N ratio for maximum PHB production were 2 g molasses with 0.3 g KNO_3 and 0.3 g yeast extract. It gave PHB percentage of 70.1 % while for potato peels optimum C : N was 4 g potato peels with 0.6 g yeast extract and maximum percentage obtained was 63.8 percent. These observations are helpful in development of an economical protocol for PHB production. Further study is required in this direction which can give useful information to achieve totally the objective of present study.

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