ISSN: 0976-3031

International Journal of Recent Scientific Research

Impact factor: 5.114

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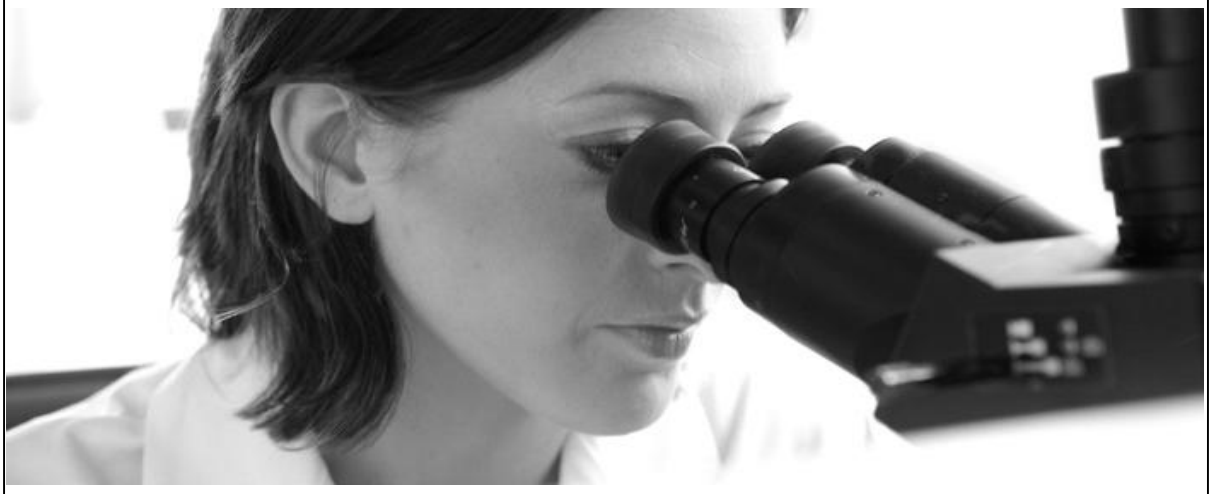

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Volume: 6 Issue: 9

THE PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH
http://www.recentscientific.com
E-mail: recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 6, Issue, 9, pp.6400-6404, September, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

EXPEDITIOUS AND ECOFRIENDLY PRODUCTION OF POLY-β-HYDROXY BUTYRATE IN BACTERIA GROWN ON RENEWABLE AGRIBYPRODUCTS

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ARTICLE INFO

Article History:

Received 05th June, 2015
Received in revised form 08th July, 2015
Accepted 10th August, 2015
Published online 28st September,2015

Key words:

Polyhydroxybutyrate, Agribyproducts, Biodegradable, Bioplastics

ABSTRACT

Bacteria producing poly-β-hydroxy butyrate thorough growth on renewable agribyproducts were optimized for expeditious PHB production. The conditions for optimum bacterial growth were manipulated in two isolates B2 and B3 for cost effective PHB production in the presence of organic C & N nutrient sources. Low cost renewable carbon and nitrogen sources like molasses, potato peels, mustard cake and cotton cake were incorporated to increase the PHB production and to decrease its cost in an ecofriendly process. The expedited process of PHB production had a harvest time of only 3 days. Using glucose and fructose as carbon sources and mustard cake and yeast extract as nitrogen source, two strains B2 and B3 exhibited a maximum harvestable PHB as 0.045 g/l and 0.037g/l respectively.

INTRODUCTION

Polyhydroxyalkanoates (PHA), a family of biopolymers or biopolyesters with different structures, are the only bioplastics which are completely synthesized by microorganisms. Polyhydroxyalkanoates (PHAs) are aliphatic biopolymers that fully comply with requirements like *biobased*, *biodegradable*, *compostable* or *biocompatible* compounds to meet the criteria to make them *Green plastics.* Among all bio-based plastics, they are exclusive by being entirely produced and degraded by microorganisms naturally to CO_2 and H_2O under natural environment (Brinda *et al.*, 2015). Bacteria are the most widely studied organisms with regard to production of bioplastics. Predominantly, PHAs are synthesized by many gram-positive and gram-negative bacteria spread over at least 75 different genera. PHAs extracted from bacterial cells show material properties that are comparable to polypropylene (Braunegg *et al.*, 1998). PHB is the first PHA to be discovered and therefore the most studied. In their metabolism, three biosynthetic enzymes worked to convert acetyl-coenzyme-A (acetyl-CoA), into PHB. The first reaction is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by b-ketoacyl CoA thiolase (encoded by phbA). The second reaction consists of the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Finally, the (R)-3-hydroxybutyryl- CoA monomers are polymerized into PHB by P(3HB) polymerase, encoded by *phbC* (Huisman *et al.*, 1989). Although a lot of expectations have been pinned on bioplastics, but many aspects have to be dealt with to make the future of these biopolymers commercially feasible. Most important of all is the low cost achievability and a production cycle or the time taken by the bacteria for each PHB production cycle. So, there is an essential need to develop a cost effective and expeditious protocol for maximization of PHB production.The two significant causes increasing the production cost are the substrate used for the polymer production and the downstream processes. The use of low cost carbon and nitrogen sources from agribyproducts would bring down the polymer cost substantially. Other cost reduction strategies may be use of municipal wastes, banana pseudo stem, damaged food grains, pea shells, apple pomace and palm oil mill effluent, potato peels, rice bran, cotton cake and mustard cake. It was reported that microorganisms can utilize the potato processing waste (Rusebdu and Sheppard, 1995, Umesh 2009) and biowaste (Jacobus, 2001) as carbon source for production of polyhydroxyalkanoates. Gulab *et al.* (2013) studied the production of poly *β*-hydroxybutyrate (PHB) by *Bacillus*

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subtilis NG220 which utilized the sugar industry waste water supplemented with various carbon and nitrogen sources. The objective of the present study was to expedite and maximize the production of polyhydroxybutyrate by bacteria using agribyproducts and/or household or waste as carbon and nitrogen sources for the development of an economical protocol.

MATERIALS AND METHODS

Optimization of growth conditions for maximum PHB production: Bacterial strains were isolated from water and soils of diverse origin like oil spilled soil, cropped lands, sewage water, garden soil and contaminated sites. One mg of different soil and water samples were dissolved in 1 ml of sterile distilled water and serial dilutions were made 5-6 times. 100 microliter of samples from these dilutions were taken and spread on plates containing TY media. Bacterial colonies appeared after 24 hrs. A single colony was taken from the plate and streaked on a fresh TY plate for isolation of pure colony. The process of streaking is repeated several times for obtaining the single colony. The isolated pure cultures were stored at 4ºC in plates containing GSY media. For screening the PHB producing bacterial isolates Nile Blue A staining method (Ostle and Holt, 1982) was used. Nile Blue A stain was added to the autoclaved minimal medium at the final concentration of 25 µg/ml were screen under UV light after 3 or 4 days of incubation. The PHB producing strains gave an orange/yellowish color florescence. Extent of florescence indicates quality of PHB.

Combinations of agribyproducts in media for maximization of PHB: Twenty six different media were investigated to determine the suitable composition supporting maximum PHB accumulation by B3 and B2 strains. One liter of media inoculated with equal starter culture for media optimization studies and incubated for 72 and 96 hrs for B2 and B3 strains respectively. Different organic and inorganic carbon and nitrogen sources along with cheap and renewable agribyproducts were used to optimize the culture conditions for maximum PHB production**.**

Extraction of PHB: Determination of the amount of PHB was performed by sodium hypochlorite hydrolysis method (Law and Slepecky, 1961). Cell mass is collected by centrifuging bacterial culture broth at 8000 rpm. The cell mass collected from one liter broth was treated with 10 ml sodium hypochlorite (4%) by vortexing it and samples were boiled for five to ten minutes in boiling water bath. After cooling add 20 ml of chloroform to the boiled samples. Keep it at 65ºC. Chloroform evaporates and dissolved PHB recovered. 50 µl of dissolved PHB added to 2 ml of sulphuric acid and then incubated for one day at 65ºC. After that the spectrophotometer reading is taken at 235 nm.

PHB production kinetics: The progress of PHB production is determined during the growth cycle in terms of time after inoculation during the entire growth cycle. Kinetics of intracellular PHB synthesis was studied in bacterial isolates B2 and B3 using one litre of minimal medium and incubated at 37^0C. Samples were drawn at incremental time intervals spread over several days and the amount of PHB was extracted using chloroform and sodium hypochlorite method and quantified by crotonic acid estimation and dried PHB estimation.

RESULTS AND DISCUSSION

Attempts to make the PHB production cost effective and expeditious, growth conditions of bacterial isolates were optimized to develop an economical protocol for PHB production. Diverse formulations of media containing carbon and/or nitrogen from agri-byproducts were utilized as supplements in place of conventional expansive carbon and nitrogen sources. Growth of two strains on various media modifications are given in table 1 and table 2 with their relative PHB yields. Keeping Minimal media as the control, several component substitutions were attempted to maximize PHB production. The optimum conditions for maximum PHB productions were found when modified minimal media were used having the following compositions: Fructose (3g), mustard cake (2g), Yeast Extract (0.2g), K_2HPO_2 (0.6 g), $MgSO_4.7.H_2O$ (0.05 g) and $CaCl_2$. $2H_2O$ (0.02g) and another media with glucose (3g), mustard cake (1.2g), yeast extract (0.2g), K_2HPO_2 (0.6g), $MgSO_4.7.H_2O$ (0.05g) and $CaCl_2.2.H_2O$ (0.02g).

PHB production Kinetics

As shown in fig. 1, it was observed that strain B3 gave the maximum PHB production in just three days while strain B2 peaked to maximum PHB production in 4 days. These strains formed part of an expedited process for PHB production towards bioplastics to be used in blends with polymer plastic, to make them less hazardous.

Specific Carbon Source was instrumental for maximum PHB production

Molasses were used as carbon source substituting glucose in the media. In B2 strain, the crotonic acid formation (fig 2) was 51% more than glucose and 65% more than that of fructose. Crotonic acid formation with carbon source molasses also showed 54.5% more PHB than glucose and 63.3% more than fructose in case of strain B3. So, from both the isolates, B2 strain was found to be a significantly higher PHB producer as compared to B3 strain. Amongst different carbon sources, molasses (3gm/l) was found to be the best carbon source for PHB production. The next capable carbon source was glucose and fructose. Strains of *Rhizobium japonicum species* (*Cicer*), *Bradyrhizobium japonicum* USDA 110 and *Rhizobium* species 2426 produced 0.285 g/l PHB (Rusendi *et al*, 1995). Another study showed the growth of *Alcaligenes eutrophus* on ammonical nitrogen and cane molasses (Wilinson *et al*, 1967) yielded up to 26.5 % PHB of dry cell weight. Bacillus megaterium ATCC6748 produced maximum PHB ((43% w/w, dry matter) when 4% molasses and 4% CSL were used, whereas the highest biomass (7.2 g l-1) was obtained at 4% molasses and 6% CSL (Chaijamrus and Udpuay, 2008). In this light B3 was faster PHB producer per unit time while B2 was higher PHB yielder.

Nitrogen Source Impacts PHB production: Fig. 3 presents the effects of different nitrogen sources on PHB biosynthesis by two strains. The crotonic acid formation in B2 strain with nitrogen source KNO_3 was found to be 30% more than yeast extract and 23% more than cotton cake. In case of B3 strain yeast extract was the best nitrogen source which gave 50% more crotonic acid formation than KNO_3 and 30% more than that of cotton cake. From both the isolates B3 was found to be a significantly higher PHB producer compared to B2. Amongst different nitrogen sources, potassium nitrate (1gm/l) for B2 strain and yeast extract for B3 were found to be the best nitrogen sources for PHB production. The next promising and cheap nitrogen sources were cotton cake and mustard cake. Maximum PHB production (0.052 g/l) was observed in bacterial isolates *Pseudomonas aeruginosa* and *Rhizobium spp* when potassium nitrate was used as the nitrogen source (Maheshwari and Rathimalar, 2011). When cotton cake and mustard cake was used as main nitrogen source to replace KNO_3 in minimal media the PHB production were 65.9 % and 61.8% respectively (Umesh *et al.*, 2009).

Cost effective PHB production from agri-byproducts: Different carbon sources from agri-byproducts and conventional carbon sources such as molasses, potato peels, fructose, glucose and their combination along with defined nitrogen source were used for comparison of PHB production. Molasses (3g) was found as better PHB producing carbon source by comparing crotonic acid and dry weight of PHB produced. It was observed that Potato peels + molasses was found as better carbon source combination for PHB production than potato peels + glucose.

The dry PHB weight was approximately 16% more and crotonic acid formation was 19% more when potato peels + molasses were used as carbon source along with KNO_3 (table 1). But the wet cell pellet weight was only 4% more when potato peels + molasses were used as carbon source. Thus combination of agri- byproducts as a carbon sources proved as better carbon source than conventional carbon sources employed.

In B3 strain, It was found that when mustard cake was used in combination of KNO_3 as nitrogen source, cell pellet was 35.6% more than glucose+mustard cake and 13.2% more than fructose+mustard cake+Yeast Extract. Crotonic acid estimation showed that when mustard cake+ KNO_3 were used in combination, then it was 24.4% more than mustard cake when it was used alone 7.6% more than mustard cake +yeast extract. Dry weight of PHB was about 10.7% more when mustard cake+YE used in combination than the mustard cake alone and 9.6% more than mustard cake+ KNO_3.

So, it was found that in case of B3 strain, nitrogen sources mustard cake and yeast extract were better nitrogen sources than cotton cake and KNO_3.It was also observed that when molasses was used as a carbon source in combination with nitrogen sources KNO_3 and yeast extract, then the cell pellet was 25% more than Potato peels +fructose+YE and 27.2% than potato peels + glucose+ KNO_3 whereas crotonic acid formation was only 9.4% more than Potato peels +fructose+YE and 27.2% more than potato peels + glucose+ KNO_3.

On the dry weight basis it was found when molasses were used in media as carbon source, the PHB weight was 32.1% more than Potato peels +fructose+YE and 50% more than potato peels + glucose+ KNO_3 So, molasses was best and cheap carbon source when used in combination with potassium nitrate and yeast extract. Thus combination of agri- byproducts as a carbon sources proved as better carbon source than conventional carbon sources employed.

Table 1 PHB production using different combinations of carbon and nitrogen sources with agribyproducts in B2 strain

S. No.	Carbon Source	Nitrogen source	Pellet weight (g/l)	PHB recovery (crotonic acid)	PHB wt. (mg/l)
1.	Fructose(2g)+ potato peels (1g)	KNO3 (0.65g)	1.968	0.626	19
2.	Fructose(2g)+ potato peels(1g)	Yeast Extract (1g)	1.762	0.428	15
3.	Glucose(2g)+ potato peels(1g)	KNO_3 (0.65g)	1.669	0.986	19
4.	Potato peels(2g) + molasses(1g)	KNO_3(0.65g)	1.403	0.465	3
5.	Potato peels(2g) + molasses(1g)	Yeast Extract (1g)	2.159	0.651	7
6.	Potato peels(2g) + glucose(1g)	Yeast Extract (1g)	1.240	0.757	9
7.	**Molasses(3g)**	**KNO_3 (0.3g)+ Yeast Extract (0.1g)**	**2.705**	**0.974**	**28**
8.	Molasses(3g)	KNO_3 (0.65g)	2.511	0.462	6
9.	Molasses(3g)	Yeast Extract (1g)	3.507	0.347	3
10	Molasses(3g)	Mustard cake(1.2g)+ KNO_3 (0.3g)	3.195	0.575	6
11	**Molasses(3g)**	**Mustard cake(2g)**	**2.851**	**1.039**	**34**
12	Glucose(3g)	Cotton cake(2g)	3.484	0.476	5
13	Glucose(3g)	Mustard cake(2g)	1.507	0.538	4
14	Molasses(3g)	Mustard cake(1.2g)+Yeast Extract(0.3g)	1.614	0.629	12
15	Molasses(3g)	Cotton cake(1.2g)+Yeast Extract(0.1g)	1.965	0.357	2
16	Molasses(3g)	Cotton cake(1.2g)+ KNO_3 (0.1g)	1.643	0.563	7
17	Molasses(3g)	Cotton cake(2g)	1.312	0.494	6
18	Glucose(3g)	Mustard cake(1.2g)+ KNO_3(0.1g)	1.478	0.853	8
19	Glucose(3g)	Cotton cake(1.2g)+ KNO_3(0.1g)	2.851	1.395	26
20	**Glucose(3g)**	**Mustard cake(1.2g)+ Yeast Extract (0.2g)**	**2.667**	**1.075**	**33**
21	Glucose(3g)	Cotton cake(2g)+ Yeast Extract(0.2g)	2.195	0.796	22
22	Fructose(3g)	Cotton cake(2g)+ Yeast Extract(0.2g)	2.225	0.762	20
23	Fructose(3g)	Mustard cake(2g)+ Yeast Extract(0.2g)	1.410	0.959	26
24	**Glucose(3g)**	**Mustard cake(1.8g)+ Yeast Extract(0.8g)**	**3.210**	**0.946**	**37**

Table 2 PHB production using organic and inorganic carbon from agri-byproducts along with organic and inorganic nitrogen in B3 strain

S. No.	Carbon Source	Nitrogen source	Pellet weight (g/l)	PHB recovery (crotonic acid)	PHB wt. (mg/l)
1.	Fructose(2g)+potato peels(1g)	KNO3 (0.65g)	1.968	0.986	19
2.	Fructose(2g)+potato peels(1g)	Yeast Extract (1g)	2.022	0.725	14
3.	Glucose(2g)+potato peels(1g)	KNO_3 (0.65g)	1.449	0.492	8
4.	Potato peels(2g)+molasses(1g)	KNO_3(0.65g)	2.196	0.653	5
5.	Potato peels(2g)+molasses(1g)	Yeast Extract (1g)	2.446	0.454	2
6.	Potato peels(2g)+glucose(1g)	Yeast Extract (1g)	2.067	0.761	11
7.	Molasses(3g)	KNO_3 (0.3g)+YE(0.1g)	2.705	0.893	28
8.	Molasses(3g)	KNO_3 (0.65g)	2.511	0.462	6
9.	Molasses(3g)	Yeast Extract (1g)	3.507	0.347	3
10	Molasses(3g)	Mustard cake(1.2g)+ KNO_3 (0.3g)	2.565	0.881	28
11	Molasses(3g)	Mustard cake(2g)	3.568	0.481	19
12	Glucose(3g)	Cotton cake(2g)	0.498	0.544	21
13	Glucose(3g)	Mustard cake(2g)	1.651	0.661	25
14	Molasses(3g)	Mustard cake(1.2g) + Yeast Extract(0.3g)	1.024	0.691	21
15	Molasses(3g)	Cotton cake(1.2g)+Yeast Extract(0.1g)	1.281	0.482	7
16	Molasses(3g)	Cotton cake(1.2g)+ KNO_3 (0.1g)	1.041	0.524	20
17	Molasses(3g)	Cotton cake(2g)	1.240	0.750	8
18	Glucose(3g)	Mustard cake(1.2g)+ KNO_3(0.1g)	1.312	0.736	6
19	Glucose(3g)	Cotton cake(1.2g)+ KNO_3(0.1g)	0.719	0.839	20
20	Glucose(3g)	Mustard cake(1.2g)+ Yeast Extract(0.2g)	0.850	0.865	25
21	Glucose(3g)	Cotton cake(2g)+ Yeast Extract(0.2g)	3.108	0.757	23
22	Fructose(3g)	Cotton cake(2g)+ Yeast Extract(0.2g)	2.215	0.664	23
23	Fructose(3g)	Mustard cake(2g)+ Yeast Extract(0.2g)	2.225	0.814	31
24	Fructose(4g)	Mustard cake(1.5g)+ Yeast Extract(1.5g)	3.557	0.925	45

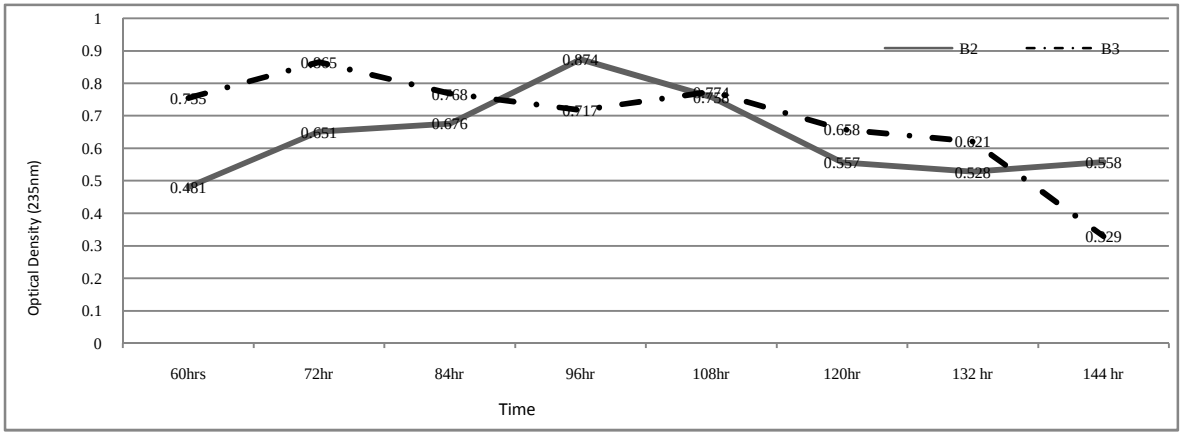

Fig 1 Kinetics of PHB production in two strains expressed as crotonic acid (O.D. 235)

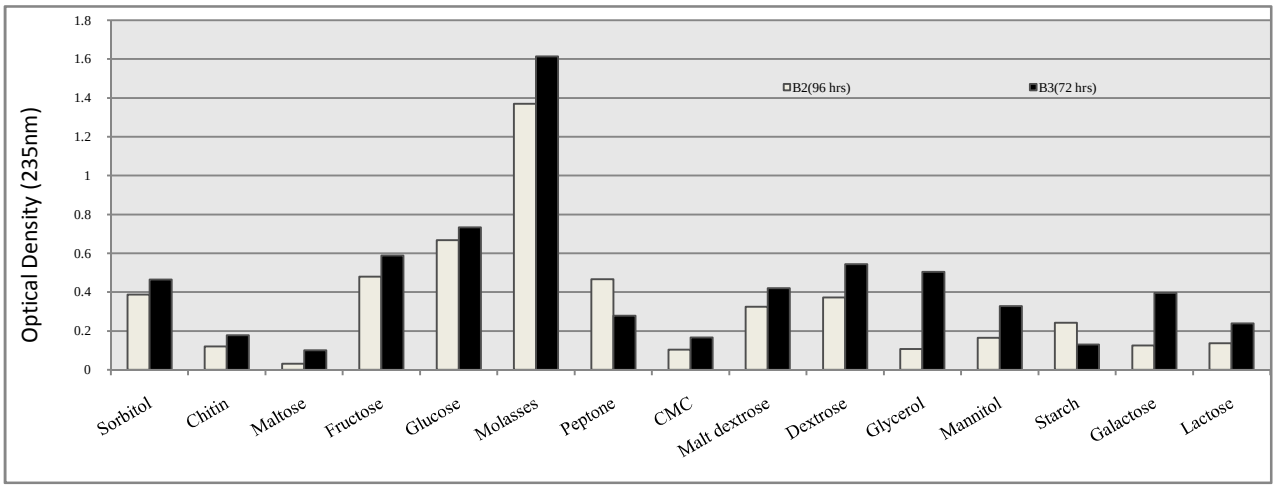

Fig 2 Effect of different carbon sources on PHB (expressed as crotonic acid (O.D. 235)

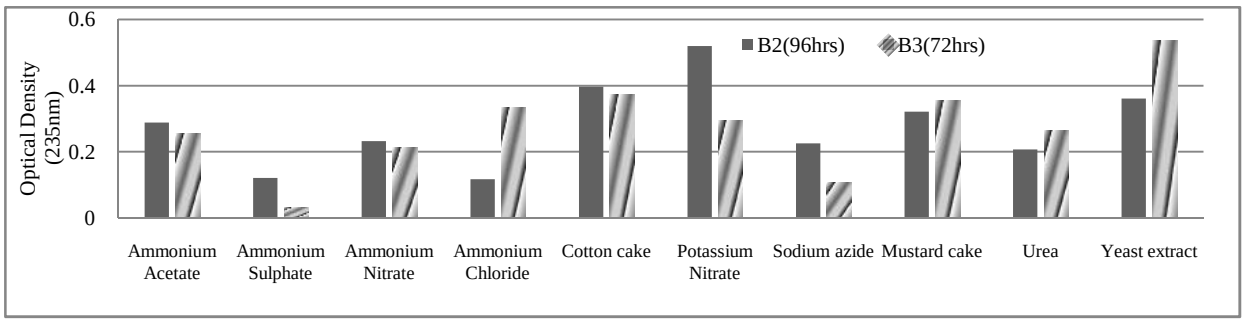

Fig 3 Effect of different nitrogen sources on PHB (PHB expressed as crotonic acid (O.D. 235)

CONCLUSIONS

The critical evaluation of the results on PHB production kinetics in bacterial isolates B2 and B3 showed that strain B3 gave maximum PHB production at just three days and strain B2 gave maximum PHB production in 4 days. All the scale up experiments of PHB extraction the biomass was harvested after 72 h. for B3 and 96 h for B2. These strains helped us to develop a expedited protocol for PHB production, from renewable agribyproducts, as bioplastics to be used later in blends with polymer plastic, to make them less hazardous. In this study, a total of twenty four different media combinations were investigated to determine the suitable composition supporting maximum PHB accumulation by B2 & B3 strains. Based on the results of several of these experiments, for B3 strain carbon source fructose when used in combination with nitrogen source mustard cake and yeast extract and for B2 strain carbon source glucose when used in combination with nitrogen source mustard cake and yeast extract have given the maximum PHB yields. The efforts undertaken during these set of experiments have enabled to arrive at an ecofriendly and farmer friendly method to be adopted at large scale for making PHB a viable commercial venture. This approach is expected to greatly improve the production of PHB by bacteria which offers greater flexibility in the use of various renewable resources, culture conditions, expeditious production and higher productivity of PHB. Consequently, progressive efforts have been done in this study for searching new bacterial strains, creating new types of optimized conditions and tailoring various kinds of media to reduce the ultimate cost of production. Future efforts in the direction of adopting these processes may help in making agro PHB the bioplastics of farmers.

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How to cite this article:

Mukesh Jangra and Virendra K. Sikka.2015, Expeditious And Ecofriendly Production of Poly-β-Hydroxy Butyrate In Bacteria Grown On Renewable Agribyproducts. *Int J Recent Sci Res*, 6(9), 6400-6404.

International Journal of Recent Scientific Research

ISSN 0976-3031