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PHB Production from Dairy Industry Soil Isolates using Whey as Carbon Source

Dr. Nausheen Sadaf[1] and Prof. Dr. D.C. Kamthane[2]
Lecturer, Department of Biology, Times Public Junior College, Udgir, Maharashtra, India[1]
Head & Research Guide, Dept.of Microbiology, Shri Guru Buddhiswami College, Purna, Maharashtra, India[2]
nausheen.sadaf@rediffmail.com, daiwa.kamthane@rediffmail.com

Abstract: *PHB producing bacterium was isolated from dairy industry soil. Identification was performed by Sudan black B and Nile blue A staining. PHB production was performed by using whey as sole carbon source with minimal medium. Comparative production using pure sugars as carbon source was also carried out. Molecular characterization of most efficient producer was done by 16S rRNA sequencing. The strain was identified as Bacillus cereus (NCBI Accession number- MZ605040). Effect of different parameters on production was also carried out and it was found that maximum production (54%) takes place with 3% of whey at pH 7 and temperature 35°C after 48 hours. On comparison with pure sugars, efficient production was observed with whey. The formed PHB was initially confirmed by UV-VIS spectrophotometry with maximum absorbance at 235nm confirmed by FTIR, GCMS, LCMS, HPLC, DSC, 13C- NMR, 1H-NMR. Biodegradation studies of produced polymer were also carried out and the polymer was found to be completely biodegradable in both in vivo and in vitro conditions. The present investigation aims to isolate and identify potent PHB producers on a cheap and easily available carbon source that is whey.*

Keywords: PHB, 16S rRNA Sequencing, Bacillus cereus, FTIR, GCMS, LCMS, DSC, ^{13}C- NMR, ^{1}H-NMR.

I. INTRODUCTION

PHA is polyesters that are naturally or artificially accumulated as water insoluble granules within a variety of microorganisms, subject to specific conditions. PHAs are regarded as a renewable resources-based alternative to petrochemical polymers. The advantageous character of PHAs lies in its environmental biodegradability and bio compatibility. PHAs cover a large scale of biological polyesters having properties in the range from thermoplastic to elastomers. The first identified and still most investigated PHA is poly-3-hydroxy butyrate (PHB) (Kovalcik et al. 2019, Devi et al. 2015 and Mohapatra et al. 2015).

Poly-L-hydroxybutyrate (PHB) is thermoplastic polyester. It is biocompatible and biodegradable, and therefore, of industrial interest. In the cell, PHB is an intracellular storage material synthesized during unbalanced growth conditions. All bacteria which are capable of PHB synthesis accumulate PHB during the stationary phase of growth when the cells become limited for an essential nutrient but have an excess for carbon sources (Aslim et al. 1998, Ghate et al. 2011 and Reddy & Thirumala 2012).

II. MATERIALS AND METHODS

2.1: Collection of Samples

The soil samples were collected from Nanded Dairy situated in the MIDC area.

2.2: Isolation of Bacteria

The samples were serially diluted and used as inoculum for streaking on nutrient agar plates. The colonies showing positive results for Sudan black B staining and Nile blue staining were taken forward for PHB production and identification by microbiological tests according to Bergey's Manual ((Abdel Kareem et al. 2017).

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2.3: Screening for PHB Production

After incubation, the colonies were subjected to Sudan black B and Nile blue A staining. The colonies showing the presence of accumulation of granules were used to proceed further (Bhuwal et al. 2013, Bhuwal et al. 2014, Reddy & Thirumala 2012).

2.4: PHB Production with Pure Sugars

For pure sugars, the strains were inoculated in minimal media supplemented with 2% lactose and for crude source, medium was supplemented with 2% whey. The flasks were incubated for 24hrs, 48hrs, and 72hrs at 37°C.

2.5: PHB extraction & Confirmation

PHB extraction was carried out by Abdel Kareem et al. 2008 and Aguirre et al. 2017 method.
The cells were lysed with NaOH digestion method and centrifuged at 6000rpm for 20min.
The extracted PHB was confirmed by UV-VIS Spectrophotometer. In this method, the PHB was acidified with concentrated sulphuric acid to form crotonic acid which was measured at 235nm.

2.6: Molecular Characterization

Molecular characterization was performed by using techniques FTIR, ^{1}H-NMR and ^{13}C-NMR, HPLC, GCMS, LCMS and DSC (Bhuwal et al. 2013, Bhuwal et al. 2014).

2.7: Biodegradation studies

A. In-vitro biodegradation of PHB

The PHB degrading ability of fungal and bacterial cultures in solid medium was determined by over layer plate assay, and in liquid medium was determined by measuring turbidity in growing culture having PHB as sole carbon source.

B. In-vivo biodegradation of PHB

Biodegradation of PHB biofilms: PHB biofilms will be prepared using chloroform as solvent as per the method described by Nadia Altaee et al and subjected to biodegradation.
(Gangurde et al. 2017, Ramchander Merugu 2012, Kumaravel et al. 2010, Altaee et al. 2016, Singh et al. 2013, Tansengco & Dogma 2004 and Rech et al. 2020).

III. RESULTS

3.1: Identification of PHB Producing Bacteria

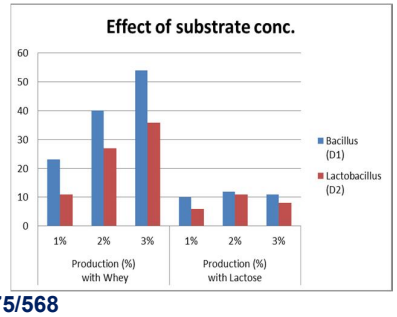
In Sudan black B staining, Purple to black granules were observed intracellular with pink background in PHB accumulating bacteria. The PHA-accumulating colonies, after Nile blue A staining, showed strong bright fluorescence on irradiation with UV light.

3.2: PHB Production

The bacterial strains that showed positive results for Sudan black B and Nile blue A staining were selected for PHB production. The production media were supplemented with crude carbon sources and a comparative set with pure sugars was also set up.

- Effect of substrate concentration on PHB production by the isolates from dairy industry soil

	Production (%) with Whey			Production (%) with Lactose		
	1%	2%	3%	1%	2%	3%
Bacillus (D1)	23	40	54	10	12	11
Lactobacillus (D2)	11	27	35.8	6	11	8



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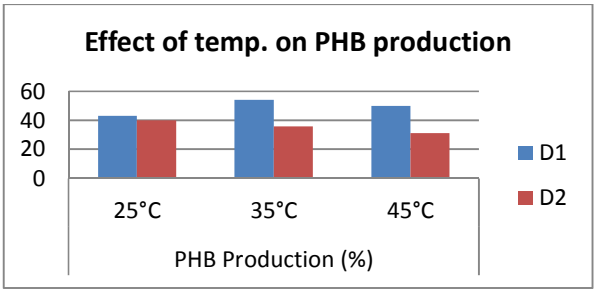
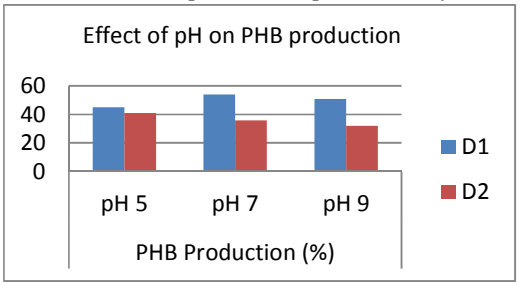
- Effect of temperature on PHB production by isolates from dairy industry soil



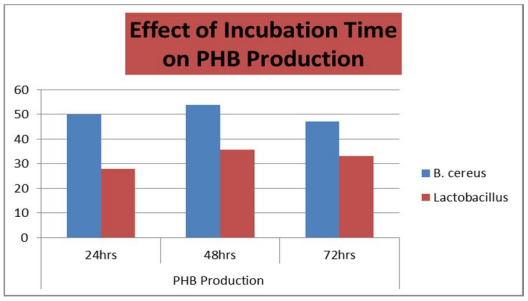
	PHB Production (%)		
	25°C	35°C	45°C
B. cereus (D1)	43	54	50
Lactobacillus (D2)	40	35.8	31

- Effect of pH on PHB production by isolates from dairy industry soil



	PHB Production (%)		
	pH 5	pH 7	pH 9
B. cereus (D1)	45	54	51
Lactobacillus (D2)	41	35.8	32

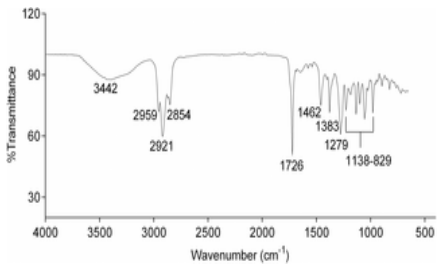
- Effect of incubation time on PHB production by isolates from dairy industry soil

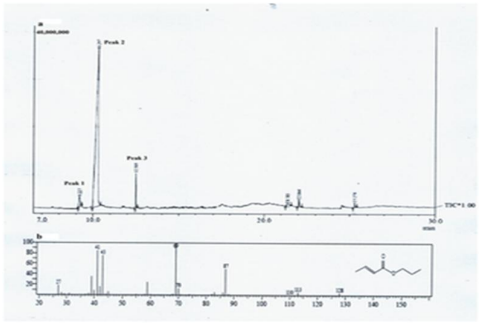


Strain	PHB Production(%)		
	24hrs	48hrs	72hrs
B. cereus(D1)	50	54	47
Lactobacillus (D2)	28	35.8	33

3.3: Molecular Characterization

FTIR GCMS





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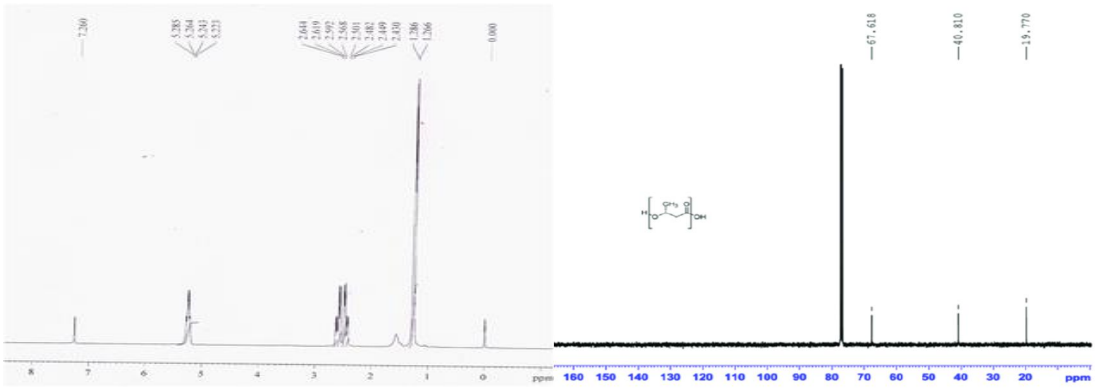
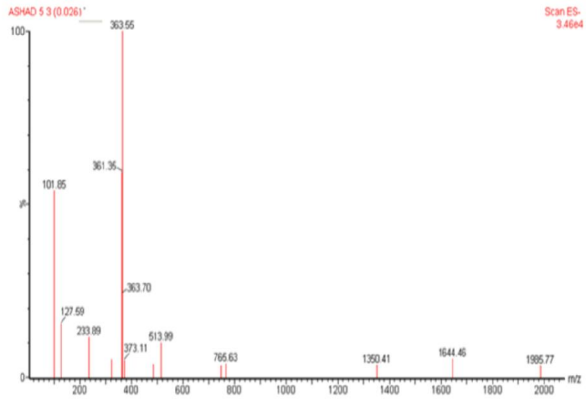
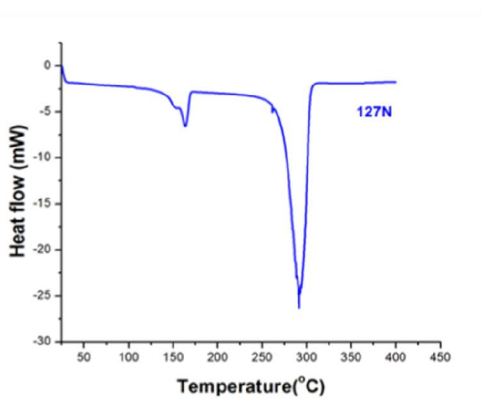
^{1}H-NMR ^{13}C-NMR

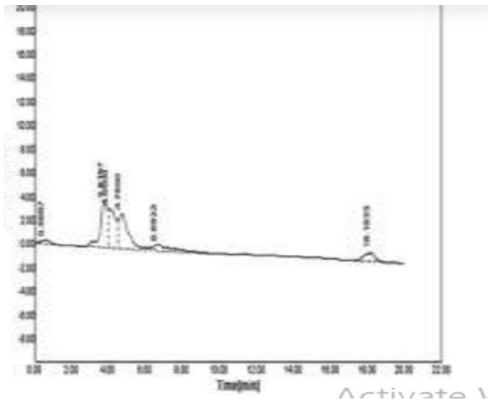


LCMS DSC





HPLC



3.4: Biodegradation Studies

a) Biodegradation in liquid and solid medium by soil isolated bacteria

The biodegradation studies were carried out in minimal medium supplemented with 01.g/ml PHB as sole carbon source. Isolated strain of *Bacillus* was inoculated in it.

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b) Invivo Biodegradation Studies

The PHB films of definite size were buried in soil. The changes were observed after every week. The observed changes were in the form of reduction in size, decomposition, holes and cracks that could be related to their biodegradation by soil microbes.

IV. CONCLUSION

From the results, it can be concluded that PHB producing bacteria can be found in Dairy soil. The PHB can be produced by using crude sources like whey that gives more production (54%) than on pure sugars.

Moreover, the most appropriate parameters for PHB production are 3% of whey at pH 7 and temperature 35°C after 48 hours.

The produced PHB was confirmed using techniques like FTIR, HPLC, GCMS, ^{1}H-NMR, ^{13}C-NMR, LCMS, DSC.

16S rRNA sequencing of most promising producer D1 shows it is *Bacillus cereus* NCIM- MZ605040.

In this way, the present study concludes that *Bacillus cereus* isolated from dairy soil shows maximum PHB production (54%) on whey as compared to pure sugars. The produced PHB was confirmed with different techniques like FTIR, GCMS, LCMS, HPLC, DSC, ^{13}C- NMR, ^{1}H-NMR.

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