*Eco. Env. & Cons. 29 (1) : 2023; pp. (141-146)*
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*ISSN 0971–765X*

**DOI No.: http://doi.org/10.53550/EEC.2023.v29i01.022**

# Isolation and Characterization of Polyhydroxybutyrate (PHB) Producing Microorganisms from Karad Region, Maharashtra

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(Received 11 July, 2022; Accepted 31 September, 2022)

**ABSTRACT**

World population is increasing day by day and to satisfy their various needs, plastic material become part and parcel of life. Millions of tons of these non degradable plastics accumulate in the environment per year and cause hazardous effects on flora and fauna. Hence there was ever pending search for bioproduct which can substitute this nonbiodegradable plastic. Polyhydroxybutyrates (PHB) are drawing much attention as they are biodegradable, environmentally friendly, and also biocompatible bioplastics. In the present study an attempt was made to isolate and characterize the PHB producing microorganisms from Karad region of Maharashtra. Total 75 isolates obtained from soil, spent wash, pond water, dairy effluent, cow dung, soil and organic waste samples were enriched using mineral salt medium and then screened primarily for PHB producers by Sudan Black B plate staining method followed by secondary screening using Nile blue plate assay method. As a result of screening, 11 PHB producing potential isolates were obtained which were then studied for morphological, biochemical and enzymatic characters. By referring Bergeys Manual of Determinative Bacteriology and 16 S r RNA technique these 11 PHB producing isolates were identified and found from genera *Bacillus, Candida, Citrobacter* and *Klebsiella, Staphylococcus* and *Rhodococcus.*

***Key words*** *: PHB producers, Sudan Black B, Nile blue, Bioplastic, PHA*

## Introduction

Plastic materials which is a part and parcel of our daily lives are now causing serious environmental problems. Millions of tons of these nondegradable plastics accumulate in the environment per year and cause hazardous effects on flora and fauna. Hence posing environmental burning problem across the world. Unlike petrochemical-based plastics that take several decades to fully degrade, PHAs can be completely degraded within a year by variety of microorganisms into $CO_2$ and water.

PHAs are carbon and energy reserve polymers produced in some microorganisms when carbon source is in plentiful and other nutrients such as nitrogen, phosphorus, oxygen or sulfur are limited. Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoic acid (PHA), a biodegradable plastic produced by microorganisms first discovered by Lemoigne (1926).

Polyhydroxyalkanoic acids (PHAs) are common intracellular compounds found in archaea, bacteria and in few eukaryotes such as yeasts and fungi (Peters and Rehm, 2005). Among the members of PHA family, Polyhydroxybutyrate (PHB) belong to polyester class. PHB is a fully biodegradable polyester,

partially crystalline material with a high melting temperature. PHB is having optical activity, piezo-electricity, and very good barrier properties and promising alternative to synthetic nondegradable plastics. (Pan and Inoue, 2009; Kabe *et al*., 2012) Polyhydroxybutyrates (PHBs) act as energy reserves in different micro-organisms such as *Alcaligenes, Staphylococcus spp, Algae. Ralstoniaeutrophes, Azotobacter beijerinckia, Bacillus megaterium, Pseudomonas oleovorans* and various nitrogen fixing microorganisms found in root nodules of legumes plant family (Haywood *et al*., 1988; Peoples and inskey, 1989).

These polymers are accumulated as intracellular membrane enclosed inclusion up to 90% of the cell dry weight under conditions of nutrient stress and act as energy reserve material. PHB can be used in the manufacture of pots, spoons, and plastic bags, among other objects, and used to make heteropolymers with other synthetic polymers and many more applications in agriculture, packaging and medical field being biodegradable and also immunologically compatible with human tissue and possess properties similar to polypropylene (Gao *et al*., 2011).

Taking in to consideration the significance of the PHB producing organisms as a suitable bioplastic producer and never-ending search for newer microorganisms having such potential, the present study was aimed for isolation and characterisation the PHB producers from unexplored Karad region.

## Methods

### Collection of samples

Samples such as soil, spent wash, pond water, dairy effluent, cow dung, soil and organic waste samples from Karad region (Latitude: 17.286501, Longitude : 74.181427 ) were used as source for isolation of microorganisms. Lagoon samples were collected from Jayawant Sugar Ltd. Dhawarwadi Karad, dist Satara while dairy samples were collected from Koyana Dudh Sangh, Khodashi Karad. Out of total eleven samples, three samples of soil, two samples each of pond water, organic waste and dung while one sample each of spent wash and dairy were collected in screw capped or zip-lock cover using spatula, transported to laboratory, maintained at ambient temperature and were then used in the present study.

### Enrichment and Isolation of microorganisms

Samples were enriched separately in mineral salt medium (g/l of urea 1.0, yeast extract 0.16, $KH_2PO_4$ 1.52, $Na_2HPO_4$ 4.0, $MgSO_4$ $7H_20$ 0.52, $CaCl_2$ 0.02, Glucose 40, Trace elements $ZnSO_4$ $7H_2O$ 0.13, $FeSO_4$ $7H_2O$ 0.02, $(NH_4)_6MO_70_{24}$ $4H_2O$ 0.06, $H_3BO_3$ 0.06) supplemented with 10% spent wash and incubating at room temperature for 24 to 48 hrs. After enrichment each sample was serially diluted in sterile distilled water up to $10^{-7}$ separately, followed by plating 1 ml of final dilution on carbon rich nutrient agar plates. Plates were incubated at ambient temperature 28-30 degree for 24-48 hrs. After incubation well isolated colonies were selected and transferred on nutrient agar slants, incubated, then preserved at refrigeration temperature and used for screening.

### Primary screening of PHB producing microorganisms

Selected isolates were inoculated on nutrient agar with 1% glucose, incubated at room temperature (28-30 degree) until colonies grow to maximum size. Then the colonies from the replica plates were flooded with 0.02% alcoholic solution of Sudan black B to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding 100% ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black. The organisms from the colonies were then subjected to Sudan black B staining and ranked based on the magnitude of their staining according to Nandini *et al.*, (2011) and Burdon (1946).

### Sudan black B staining

A smear of the suspension on a clean glass slide was fixed by heating, followed by addition of few drops of Sudan black B solution (0.3% in 70% ethanol) in order to stain them. After 20 minutes of treatment, slide was immersed several time in xylene solution and blot dried with absorbent paper, followed by addition of 0.5 % Safranin solution. The slide was washed with tap water, dried and observed under oil immersion lens of microscope for bluish black organisms (Burdon, 1946).

### Secondary screening of PHB producing microorganisms

Sudan black B positive isolates were further

screened with Nile blue. A plate assay method on carbon rich nutrient agar medium containing 0.0005 g Nile blue. The colonies of PHB accumulating strains when stained with Nile blue A show bright orange or pink fluorescence on exposure with UV light and their fluorescence intensity increases with the increase in PHA content of the bacterial cells. The isolates which showed bright orange fluorescence on exposure with UV light after Nile blue Aplate assay method were selected as PHB producers (Burdon, 1946, Cain, 1947; Kitamura and Doi, 1994).

Microorganisms showing both sudan black B and Nile blue plate assay method positive were considered as potential PHB producers.

**Study of characteristics of potential PHB producers**

All selected PHB producers were then studied for their colonial characteristics, biochemical characteristics (glucose, arabinose, maltose, sucrose, lactose and IMViC test) and enzymatic activity (amylase, caseinase, lipase, gelatinase, urease, phenyl alanine deaminase, oxidase and catalase) as per the methods of John *et al*., (2009).

**Identification of potential PHB producers**

Morphological, physiological and biochemical tests results of the PHB producing isolates were used to identify the isolates to Genus level by referring Bergey's Manual of Determinative Bacteriology (John *et al*., 2009) and promising isolates were identified by 16 S r RNA technique.

## Results and Discussion

After enrichment of eleven samples in mineral salt medium, total 75 isolates were obtained on nutrient agar supplemented with 1% glucose. Out of 75 organisms when primarily screened for ability to produce PHB granules (Fig. 1), only 35 isolates were found efficient to produce PHB granules studied by Sudan black B slide and plate staining method. From these 35 isolates 24 isolates were obtained from secondary screening on the basis of Nile blue plate assay method (Fig. 2)

Microorganisms showing both sudan black B and Nile blue plate assay method positive were considered as proper PHB producers. These were then sorted for 11 potential isolates with ability of maximum excellent PHB production

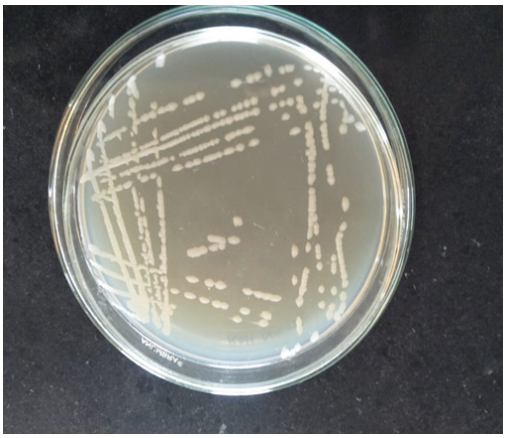

**Fig. 1.** Growth of PHB producing organisms on nutrient medium

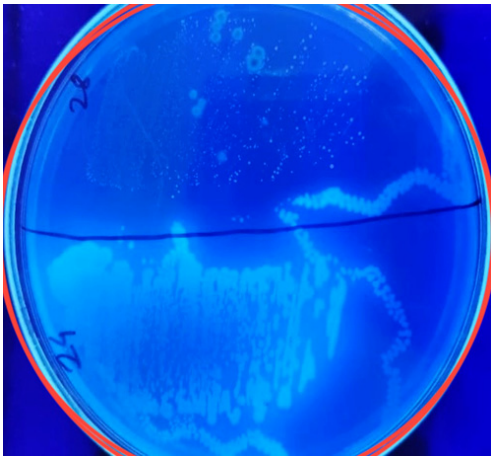

**Fig. 2.** Fluorescent growth of PHB producers by Nile blue plate assay method under U.V. illuminator.

Table 1 summarizes the colony characteristics of these 11 potential PHB producers

It was observed from the colony characteristics of these 11 organisms that there were not much differences in characteristics except few in terms of colour (Table 1).

When these 11 isolates were gram stained to study gram nature and morphology, it was observed that all were gram positive except isolates 37 and 41. It is clear from Table 2 that isolate 33, 45 and 46 were observed as Gram positive cocci, isolates 10, 20, 24, 38 and 72 were observed as Gram positive rods, isolates 37 and 41 were Gram negative rods and only isolate 1 was observed as member of Yeast.

Sudan black B plate staining method showed visual differences in bluish black colour intensity which was noticed higher in isolates 10, 20, 24, 37

**Table 1.** Colony characteristics of potential PHB producers.

| Isolate No. | Colour | Shape | Size | Elevation | Margin | Opacity | Consistency | Gram nature |
|---|---|---|---|---|---|---|---|---|
| 1 | White | circular | 1 mm | convex | entire | opaque | moist | gram +ve yeast |
| 10 | Cream white | circular | 1mm | flat | entire | opaque | moist | gram +ve rod |
| 20 | White | circular | 2-3mm | convex | entire | Opaque | moist | gram +ve rod |
| 24 | Pinkish white | circular | 1mm | flat | irregular | opaque | moist | gram +ve rods |
| 33 | White | circular | pinpoint | flat raised convex | entire | opaque | moist | gram +ve cocci |
| 37 | White | circular | 1mm | flat | entire | opaque | moist | gram -ve rods |
| 38 | White | circular | 2mm | flat | entire | opaque | moist | gram +ve rod |
| 41 | Yellow | circular | 1mm | convex | entire | translucent moist | | gram -ve rods |
| 45 | White | circular | 1-2 mm | convex | irregular | opaque | moist | Gram +ve cocci |
| 46 | Cream white | circular | 1-2mm | convex | entire | opaque | moist | gram +ve cocci |
| 72 | Pinkish white | 1mm | circular | convex | entire | opaque | moist | Gram + rods |

38, 41, 72 and slightly less in 33, 45 and 46. The ability to accumulate PHB granules were then when confirmed by sudan black B staining method. Isolates 20, 38 and 72 showed dark bluish black PHB granules (Table 2). Black PHB granules isolates were ranked relative to the '+' sign. Highly stained colonies with "++++" sign, strongly stained colonies with "+++" sign, moderately stained colonies with "++" sign, poorly stained colonies with "+" sign.

Very bright fluorescence on U V exposure was found in all isolates by Nile blue A plate assay method (Fig. 2).

Microorganisms showing both sudan black B and Nile blue plate assay method positive were considered as potential PHB producers.

Vishnuvardhan Reddy (2009) isolated and characterized PHB producing Bacillus from a municipal sewage treatment plant in Hyderabad. Kritika Sinha and Pragya Rathore (2015) isolated different PHB producing microorganisms from soil using E2 medium and screened for PHB production by Sudan Black B dye plate assay. Similarly we have also isolated PHB producing organisms from soil and used Sudan Black B dye plate assay method.

Biochemical characteristics of potential PHB producers are presented in Table 3.

Table 2 summarizes the colony characteristics of potential PHB producers.

It is observed from the Table 3 that isolates 45 fermented all sugars with production of acid and gas. Isolate 24, 41 and 72 produced only acid from all sugars. Isolate 1, 10 and 33 were reported not to ferment lactose, arabinose and maltose. Glucose is the only sugar fermented by all isolates. Indole test was reported as negative for all isolates. Methyl red was found positive for all isolates except isolate 2, 4 and

**Table 2.** Staining properties of PHB producers

| Sr. No. | Isolate No. | Gram nature | Sudan Black B Slide staining | Sudan Black B plate staining | Fluorescence on U V exposure by Nile blue A plate assay method |
|---|---|---|---|---|---|
| 1 | 1 | Gram +ve yeast | ++ | ++ | ++++ |
| 3 | 10 | Gram +ve rod | +++ | ++++ | ++++ |
| 4 | 20 | Gram +ve rods | ++++ | ++++ | ++++ |
| 5 | 24 | Gram +ve rods | +++ | ++++ | ++++ |
| 6 | 3 | Gram +ve cocci | +++ | +++ | ++++ |
| 7 | 37 | Gram -ve rods | +++ | ++++ | ++++ |
| 8 | 38 | Gram +ve rods | ++++ | ++++ | ++++ |
| 9 | 41 | Gram -ve rods | +++ | ++++ | ++++ |
| 10 | 45 | Gram +ve cocci | ++ | +++ | ++++ |
| 11 | 46 | Gram +ve cocci | ++ | ++ | ++++ |
| 12 | 72 | Gram + ve rods | ++++ | +++ | ++++ |

37. Both tests -VP and citrate were reported positive for isolate 20 and 45. Isolate number 10 showed only methyl red positive, similarly only citrate was reported positive for isolate 37.

It seen from Table 4 that amylase was produced by all isolates except isolate 1 and 41. On the other hand phenyl alanine deaminase was not produced by any isolates. Caseinase lipase gelatinase, urease activity were reported in six, four eight and five isolates respectively. Similarly oxidase and catalase activity were reported in seven and nine isolates. Thus these potential isolates showed multiple enzymatic activities, hence played role in degradation of complex compound which was later on reflected in % reduction of COD.

Using 16 S r RNA technique, isolate 1, 10, 20, 37, 41 and 72 were identified as *Candida orthopsilosis, Bacillus subtilis, Bacillus cereus, Klebsiella grimontii, Citrobacter freundii* and *Bacillus megaterium* respectively. On the other hand isolates 24, 33, 38, 45 and 46 were identified up to generic level by referring Bergey's Manual of determinative bacteriology, as isolates belonging to *Bacillus, Rhodococcus, Bacillus* and last two to *Staphylococcus.*

PHB production was achieved by *Bacillus megaterium* using dairy waste as substrate (Ram Kumar Pandian *et al*., 2010); by *Bacillus cereus* using sugar cane and beet molasses (Yilmaz and Beyatli (2005)

In the present study we have also isolated *Bacillus megaterium* and *Bacillus cereus* and confirmed their PHB production abilities.

Nayak (2012) stated that waste generated from agriculture industry, dairy industry, distillery industry, oil industry and organic waste can be used as a raw material for the production of PHA and PHB

**Table 3.** Biochemical characteristics of PHB producers

| Sr. No. | Isolate No. | Sugar fermentation | | | | | Test | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | S | G | L | AR | M | Indole | Methyl Red | V.P | Citrate |
| 1 | 1 | A | A | - | - | - | - | + | - | - |
| 2 | 10 | - | A | - | - | - | - | + | - | - |
| 3 | 20 | A | A | - | - | A | - | - | + | + |
| 4 | 24 | A | A | A | A | A | - | + | - | - |
| 5 | 33 | A | A | - | - | - | - | + | + | - |
| 6 | 37 | - | A | A | A | - | - | - | - | + |
| 7 | 38 | A | AG | A | AG | A | - | + | - | - |
| 8 | 41 | A | AG | A | A | A | - | + | - | + |
| 9 | 45 | AG | AG | AG | AG | AG | - | + | + | + |
| 10 | 46 | - | AG | - | AG | - | - | + | - | - |
| 11 | 72 | A | A | A | A | A | - | + | + | - |

S= Sucrose, G= Glucose, L=Lactose, AR=Arabinose, M=Maltose**,** A= acid, AG=Acid &gas, -= Negative test, += Positive test

**Table 4.** Enzymatic characteristics of PHB producers

| Sr. No. | Isolate No. | Production of | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Amylase | Caseinase | Lipase | Gelatinase | Urease | Phenyl alanine deaminase | Oxidase | Catalase |
| 1 | 1 | - | - | - | - | - | - | + | + |
| 2 | 10 | + | - | - | + | - | - | + | + |
| 3 | 20 | + | + | + | - | + | - | - | + |
| 4 | 24 | + | - | + | + | - | - | + | + |
| 5 | 33 | + | - | + | + | + | - | + | + |
| 6 | 37 | + | + | - | + | _ | - | + | _ |
| 7 | 38 | + | + | - | + | +(s) | - | + | _ |
| 8 | 41 | - | - | - | - | + | - | - | - |
| 9 | 45 | + | - | - | + | - | - | - | + |
| 10 | 46 | + | + | + | + | - | - | + | + |
| 11 | 72 | + | + | - | - | + | - | - | + |

**Table 5.** Identification of promising isolates:

| Sr. No. | Isolate No. | Isolate identified as | Accession Number |
|---|---|---|---|
| 1 | 1 | *Candida orthopsilosis* | OP393929 |
| 2 | 10 | *Bacillus subtilis* | OP415431 |
| 3 | 20 | *Bacillus cereus* | OP430573 |
| 4 | 24 | Member of *Bacillus* | - |
| 5 | 33 | Member of *Rhodococcus* | - |
| 6 | 37 | *Klebsiella grimontii* | OP458827 |
| 7 | 38 | Member of *Bacillus* | - |
| 8 | 41 | Citrobacter freundii | OP434597 |
| 9 | 45 | Member of *Staphylococcus* | - |
| 10 | 46 | Member of *Staphylococcus* | - |
| 11 | 72 | *Bacillus megaterium* | OP435363 |

using the microbial technology. We have also isolated PHB producing organisms from samples such as soil, spent wash, pond water, dairy effluent, cow dung, soil and organic waste

Thus our results are supported by the findings of these previous workers.

## Conclusion

Promising PHB producers can be used in dual role, first to produce bioplastic which will be a good substitute for nondegradable plastics causing hazards to flora and fauna and second for reduction of COD due to its wide range of enzymatic activities.

## Acknowledgement

We are very much thankful to the Management of the Krishna Institute for inspiration and providing all necessary laboratory facilities for the present research work

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