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BIOPOLYMER PRODUCTION (PHV) AND ITS APPLICATION TO FISHERIES

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ABSTRACT

Biodegradable plastics derived from biomass have recently gained much attention from the public because they are synthesized from renewable raw materials. PHV is one such biopolymer. The objective of this study was to isolate highest amount of PHV accumulating Bacillus sp. and to check its application in aquaculture field. Among 25 soil isolate only one isolate showed positive result for PHV accumulation by rapid screening method using Sudan black B stain. The soil isolate was identified as Bacillus subtilis by morphological, biochemical, physiological and molecular characterization, which was deposited in the genbank under accession number JQ360585. Various agro-industrial residues such as of jack fruit seed powder, rice bran, wheat bran, Whey solution and Ground nut oil cake were used as a carbon source in the production medium and the maximum PHV accumulation was observed in Whey. The extracted PHV was used to protect the aquacultures (Catla catla and Scomber japonicus) against the infection caused by Aeromonas sp. and Vibrio sp. by Invivo challenge test.

(Key words: *B.subtilis*, Agroindustrial residues, aquaculture, *Vibrio* sp, *Aeromonas* sp., Invivo challenge test)

Introduction

Synthetic plastics are easy to produce and economically feasible but it is not ecofriendly because it is recalcitrant. Accumulation of the plastics destroy aesthetic beauty of the place and the plastic present in the soil reduce the growth of roots and affect the biological balance of the soil. Plastics present in the aquatic environment kills many aquatic animals and destroy the home environment of many species. So plastics produced by the microorganisms can be used as a substitute for the petrochemically derived plastics because they are ecofriendly.

Polyhydroxyvalerate (PHV), a biodegradable plastic which is a best known member of Polyhydroxy alkanoate (PHA). It is accumulated in most of the microorganism during the stationery phase of the growth (Madison and Huisman, 1999). It is linear, unbranched and small chain polymer made up of hydroxyvaleric acid. It is a lipid and has the properties similar to petrochemically derived plastics but is biodegradable. It has got extensive application in Agriculture and Medical field. Recently it gained importance in the aquaculture field also. Aquacultures are considered important because they can meet the future global food requirement. But they are frequently infected with *Aeromonas* sp and *Vibrio* sp. which causes extensive loss to the aquacultures. Using antibiotics, lead to the development of resistant strains and developing vaccines are time consuming process and economically not feasible. To overcome these problem short chain fatty acids such as PHV can be to protect the aquacultures.

2.0 Materials and Methods

Soil samples (3.0 -4.0 cm deep from surface) were collected in and around Coimbatore for isolation of the bacteria. About 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and the plates were incubated at 30°C for 24 h. Following incubation various colonies of different morphologies were individually picked from the plates and sub cultured onto nutrient agar plates for further studies.

2.1 Rapid screening of native bacterial isolates for PHV production

Nearly 10 Bacillus cultures were qualitatively tested for PHV production following the viable colony method of screening using Sudan black B dye (Liu et al., 1998). Nutrient agar medium supplemented with 1% glucose was poured into sterile petriplates and allowed for solidification. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30°C for 24 h. Following incubation ethanolic solution of (0.02%) Sudan black B was spread over the colonies and the plates kept undisturbed for 30 min. They are washed with ethanol (96%) to remove the excess stain from the colonies, and the results were observed. From the result the soil isolate which showed positive result for PHV production was taken for further studies.

2.2 Characterization of selected soil isolates

The colony showed positive result for PHV production was taken to study the morphological, biochemical, physiological and molecular characteristics. After 24 h of incubation the colony showed positive result for PHV production were inoculated onto nutrient agar slants and incubated for 24 h. to study the morphological, biochemical, physiological and molecular characteristics. Bacterial smear was prepared on a clean slides and heat fixed. The smear was treated with crystal violet for 1 min and washed with tap water. Grams iodine was added for 1 minute, washed and alcohol was added and left for 30 s. Finally, safranin was added and left for 45 s washed, air dried and the smear was observed under microscope. Biochemical tests such as indole test, methyl red test, voges proskauer test, citrate utilization test, urease test, catalase test, oxidase test, starch hydrolysis test, casein hydrolysis test, arginine hydrolysis and tween 20 test were carried out.

2.3 Molecular characterization

16S rRNA amplification

The selected soil isolate was given for gene sequencing to Ribogen laboratory, Chennai. The primers used were 27f (5'-AGAGTTTGATCMTGGCTCAG-3') 1492r (5'TACGGYTACCTTGTTACGACTT-3'). The PCR mixtures were prepared in 50 µl volumes containing 0.5 µM of primer, Taq PCR master mix, and 1 µl of the extracted DNA. DNA amplification was performed in a thermal cycler with an initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation (0.5 min at 94°C), annealing (1 min at 50°C), and extension (1 min at 72°C), plus a final extension for 10 min at 72°C. The anticipated product of approximately 1,500 and 1,300 bp was isolated after 1% agarose gel electrophoresis of the amplified mixture using a gel extraction kit. The sequence was compared with similar 16S rDNA sequences retrieved from the DNA databases by using the BLAST search and the evolutional relationship was studied using MEGA 4 software.

2.4 Extraction and estimation of PHV by using less expensive substrates

Agro industrial residues

About 100 g of jack fruit seed powder was taken, gelatinized, liquefied and saccharified. The conditions were: gelatinization at 100°C for 15 min followed by liquefaction with alpha amylase at 85°C, pH 5 for 30 min and then saccharification with glucoamylase at 60°C for 70 min. The hydrolyzate obtained was filtered through a muslin cloth. To this, contents of nutrient broth were added and sterilized (Nisha et al., 2009).

About 2 g each of rice bran and wheat bran was taken and washed thoroughly with distilled water for 2-4 times. Then it was immersed in 125 ml of distilled water and sterilized at 121ºC for 15 min. *Aspergillus* sp. was inoculated and incubated at room temperature for 72 h. Then the fungus was separated by filtration by using Whatmann No.1 filter paper. The contents of the nutrient broth were added to 100 ml of the filtrate and sterilized. Whey solution was boiled for 5 min then filtered after cooling to remove the aggregates. The pH of the whey solution was adjusted to 7±1 by adding NaOH (12 N) then re-filtred (Yellore and Desai, 1998). The contents of the nutrient broth were added to 100 ml of the whey and sterilized at 121ºC for 15 min.

Ground nut oil cake, 50 g was taken and dissolved in 50 ml of sterile distilled water over night. Then it was filtered through Whatmann number 1 filter paper. To this filtrate contents of nutrient broth were added and sterilized.

The PHV accumulating isolate was inoculated into above mentioned media and incubated at 35ºC for 48 h. About 10 ml of the culture was taken and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 10 ml of sodium hypochlorite (Loba) and the mixture was incubated at 37ºC for 2 h. After incubation, the mixture was centrifuged at 5000 rpm for 15 min, washed with distilled water, acetone, methanol respectively and the pellet was dissolved in 5 ml of boiling choloroform. It was evaporated by pouring the solution on sterile glass tray and kept at 4ºC. After evaporation the powder was collected and weighed. The values were recorded on a graph (amount of PHV versus agro industrial waste).

2.5 Application of PHV in the aquaculture field

Invivo challenge test

Fresh water fingerlings (*Catla catla*) and marine fingerlings (*Scomber japonicus*) were collected from Koushik pet world, Coimbatore. 6 fresh water fishes and 6 marine water fishes were taken in 4 fish tanks (I, II, III and IV). 1000 mg of the PHV was added in one litre of water in the fish tanks (I and II) whereas the other 2 fish tanks (III and IV) were free of PHV (control).

After 24 h, challenge test was performed with *Aeromonas* sp. and Vibrio sp. 10 ml (10 -6 cells/ml) of 24 h old cultures of *Aeromonas* sp. and *Vibrio* sp. were inoculated into tanks I, II, III and IV respectively and observed for 1 week.

3.0 Results

3.1 Rapid screening and characterization of native bacterial isolates for PHV production

Among the 10 isolates only one isolate showed positive result for PHV accumulation and they appeared in bluish black color.

3.2 Biochemical and Physiological Characterization

For IMViC tests, the soil isolate showed negative result except for VP test indicates that they produce acetoin as an end product of sugar fermentation and cannot produce indole, methyl red and utilize citrate as a carbon source. They showed negative result for urease and positive result for catalase and oxidase. They showed positive result for all the physioloigical tests and the results are shown in the table 1.

Table 1 Biochemical and Physiological Tests

S.No	Biochemical test	B1
1.	Indole test	-
2.	Methyl red test	-
3.	Voges Proskauer test	+
4.	Citrate utilization test	-

5.	Urease test	**-**
6.	Catalase test	**+**
7.	Oxidase test	**+**
	Physiological test	
8.	Starch hydrolysis test	**+**
9.	Casein hydrolysis test	**+**
10.	Arginin hydrolysis test	**+**
11.	Tween 20 hydrolysis test	**+**

3.3 Molecular characterization

The 16SrRNA gene is used for phylogenetic studies as it is highly conserved between different species of bacteria and archaea. It also contains hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. In this present study Neighbour-Joining method (Saitou and Nei, 1987) was used to study the evolutionary history. The optimal tree with the sum of branch length = 0.17214775 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The sequence was compared with similar 16S rDNA sequences retrieved from the DNA databases by using the BLAST search.

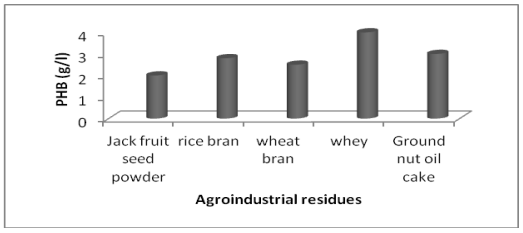
The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and the units of the number of base substitutions per site. All Morphological, biochemical, physiological and molecular characteristics indicated that the isolate was *Bacillus subtilis* and deposited in the genbank under accession number JQ360585.

3.2 Extraction and estimation of PHV using less expensive substrates.

Agro Industrial residues

Bacillus subtilis showed maximum accumulation of PHV in concentrated whey (4.0 g/l) followed by groundnut oil cake (3.0 g/l), rice bran (2.8 g/l), wheat bran (2.5 g/l) and jackfruit seed powder (2 g/l) (fig 2).

Figure 2: Estimation of PHV using Agro Industrial residues



3.3 Aquaculture Application

Invivo challenge test showed that the addition of PHV protected the fishes against the *Aeromonas* sp. and *Vibrio* sp. infection (Tanks I and II) and they remained healthy even after one week of observation whereas all the control fishes (without the addition of PHV) developed infection such as darkened colouration to the skin, fins and haemorrhage, necrosis in the intestine and died within 48 h and the results are shown in the figure 3.

Fig 3 Invivo challenge Test

Figure 12 Invivo challenge Test

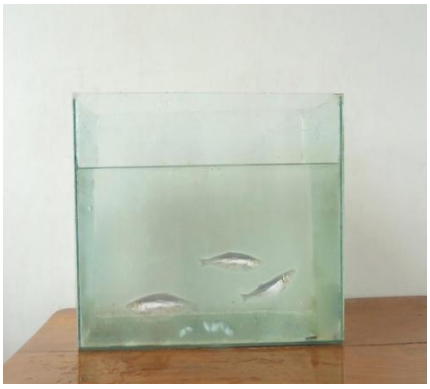
Fresh water fish *(Catla catla)*

Tank with PHB **Tank without PHB (control)**




Marine water fish (*Scomber japonicus*)

Tank with PHB **Tank without PHB (control)**

4.0 Discussion

Polyhydroxyvalerate (PHV) commonly found as intracellular granules in bacterial cells and they are synthesized during unbalanced growth condition. Both Gram positive and Gram negative bacteria have been employed for the production of PHAs.

Screening is an important procedure in the field of Biotechnology to find out the Microorganism accumulating the desirable primary or secondary metabolites. Screening by using Sudan black will give quicker result. Even though PHV is biocompatible and biodegradable, it is not completely exploited because the upstream and downstream processes are not economically feasible. Recent advances in molecular biology, low cost substrates and improvement in fermentation technology can make the production of PHV cheaper.

One such attempt is using inexpensive substrates as a carbon source in their production medium. Among the various agro-industrial residues whey showed the maximum accumulation. The main constituent of whey is lactose, both NPN (Non protein nitrogen) and casein as nitrogen sources. Even though the casein coagulation happens during milk coagulation in cheese making, some percentage of the casein will remain in the whey. The high accumulation of PHV in whey may be due lactose, NPN and casein.

The present study showed that whey can use as a carbon source in the production medium for the mass production of PHV. Similar studies were Nisha *et al*., 2009) in *Bacillus sphaericus* NCIM 5149 and Yellore and Desai 1995 using the newly isolated *Methylobacterium* sp.

Invivo challenge test showed that PHV can be effectively used to control the diseases caused by pathogens. The research data of Defoirdt et al. (2007) in *Artemia franciscana* corroborates with the result of present investigation. So finally it is concluded that PHV can be not only used as a substitute for the synthetic plastics but also can be used to protect the aquacultures against the pathogens.

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