Purification and Characterization of Polyhydroxybutyrate produced from Marine Bacteria

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Abstract

Microbial plastic Polyhydroxybutyrate (PHB) have attracted research and commercial interests because of their biodegradability and wide usage in various areas like industries, pharmacy and agriculture. Polyhydroxybutyrate accumulating bacteria CMM3 isolated from the coastal district of Andhra Pradesh and identified based on their biochemical and phylogenetic characterization. On screening with Sudan black B staining method polyhydroxybutyrate granules appeared as bluish black droplets against the pink coloured cytoplasm. Effect of various process parameters like best carbon, nitrogen sources and their concentrations, Nacl tolerance, temperature and P^H of the medium were optimized for fermentative production of Polyhydroxybutyrate. Then PHB purified from the fermentation broth by sodium hypochlorite assay method and estimated by measuring the colour intensity at 235nm using UV-Visible spectrophotometer with crotonoic acid as standard. Characterization of extracted polyhydroxybutyrate was carried out by FTIR.

Key words-Marine bacteria, Sudan black B, Fermentation, Sodium hypochlorite assay, FTIR

1 Introduction

Polyhydroxybutyrate have been drawing much attention as biodegradable substitutes for conventional non-biodegradable plastics and recommended for application in various areas like industries, pharmacy and agriculture. Synthetic plastics are produced from fossil fuels which are non renewable subjected to extinction and petrochemicals which cannot be decomposed easily. Even if degraded, the exact time of their degradation is unknown there by contributing to environmental pollution. As a consequence of excessive usage and polymeric nature of synthetic plastics, they remained persistent in the environment, posing serious threat to both terrestrial and aquatic systems. They are accumulating at the rate of 25 million tonnes / year and it is also estimated that 14 billion pounds of plastic garbage is being dumped into the world's ocean every year which is increasing steadily and severely affecting the life of living organisms. Hence, there is an immense need to degrade the polythene of the environment.

In view of this, natural materials like bioplastics are of much interest. Bioplastic is a form of plastic synthesized from renewable resources "[1]" such as plant starch and microbial species. PHB is an intracellular lipid reserve material accumulated by many bacteria under the conditions of nutrient stress. The optimal conditions for PHB production by microorganisms usually include an excess of carbon source and exhaustion of single nutrient such as Nitrogen, Phosphorous, Sulphur or Oxygen "[2]". Marine organisms have strategies to produce novel compounds like PHB due to the unique environment they survive. Hence, the present research work has been aimed to use marine microorganisms as biological tools for production of PHB.

2 Materials and Methods

2.1 Collection of marine samples:

Marine samples are collected from Chirala of Prakasam District, Andhra Pradesh at a depth of six feet from sea shore (1Km distance) and also deep sea water under aseptic conditions in polythene containers and brought to the lab to characterize their physico-chemical properties and for isolation of marine bacteria.

2.2 Physico-chemical properties of marine samples:

Various physico-chemical properties like pH, temperature, turbidity, salinity, conductivity are measured by using electronic devices. Total dissolved solids **(TDS)**, biological oxygen demand **(BOD)**, chemical oxygen demand **(COD)**, dissolved oxygen **(O_2)** content, total nitrogen **(N2)** content and total carbon **(C)** content of marine water samples was determined by following the standard procedures in the manuals **"Standard Operating Procedures for Water Sampling- Methods and Analysis"** by Department of Water, Government of Western Australia and Practical Microbiology by **Dubey and**

2.3 Isolation of marine bacteria:

Marine samples were subjected to 10 fold serial dilutions ranging from 10^{-1} to 10^{-8} and plated on marine agar medium by spread plate technique. After seven days of incubation at 37°C, microbial isolates obtained were purified, sub cultured and maintained on the respective agar slants at 4^0C.

2.4 Screening of marine isolates for PHB production:

The isolates were further screened for PHB production by Sudan Black B staining method "[3]". In Sudan black B method, microbial isolates were stained with Sudan black B (0.3%, w/v) for 5 to 15 minutes and then immersed in xylene. Finally counter stained with aqueous safranine (0.5%, w/v) for 30 seconds. The PHB granules in microbial isolates appeared as blue black droplets while the cytoplasmic part of micro organisms appear as pink under oil immersion objective lens.

2.5 Characterization of potent marine isolate:

Selected PHB producing bacterial strains stained by standard gram staining procedure for their morphological characterization. The potent isolates were observed for colony characterisation by observing the colour, shape, size and appearance of the colonies. Various biochemical tests namely Indole production, Methyl Red test, Voges Proskauer, Citrate utilization test, Urease test, Oxidase test, Catalase test, Carbohydrate fermentation, Oxidative fermentation, Starch hydrolysis and Nitrate Reduction tests were performed to know their biochemical properties.

2.6 Sequencing:

The positive marine isolate was subjected to molecular characterisation by 16s rRNA sequencing "[4]" and the resulting nucleotide sequence submitted to Genbank for the allotment of accession number.

2.7 Fermentative production of PHB:

Further we selected the positive isolate for the fermentative production of PHB by optimizing the cultural conditions as our earlier report "[5]".

Effect of Carbon Sources

The PHB producing strain, selected by primary screening were subjected to PHB production by using different carbon sources like glucose, sucrose, maltose and fructose at 1% level respectively.

Effect of Nitrogen Sources:

The PHB producing strain grown in with best carbon source and different nitrogen sources like ammonium chloride, ammonium sulphate, ammonium nitrate and yeast extract respectively.

Effect of different concentrations of best Nitrogen source

The bacterial isolate grown in a flask having the best carbon source at 1% and different concentrations of best nitrogen source *viz.*, 0.2, 0.4, 0.6, 0.8 and 1g/100 ml.

Effect of different concentrations of best Carbon source

The positive isolate grown in medium with different C: N ratios 1:0.2; 1.5:0.2; 2:0.2 and 2.5:0.2 using the best carbon and nitrogen sources.

Effect of NaCl Concentration

Different concentrations of NaCl *viz.*, 0.5, 1.0, 1.5, 2.0 and 2.5 are tested to know the optimal concentration of NaCl for PHB production.

Effect of pH and Temperature

After optimising the carbon and nitrogen sources, different pH of the media (5.0, 6.0, 7.0, 8.0) and different temperatures (20^0 C, 30^0 C, 40^0 C and 50^0 C) are tested to know the optimal conditions for PHB production.

After 48 hours of incubation PHB yields were quantified spectrophotometrically by Na hypochlorite assay method.

2.8 Purification and quantitative estimation of PHB from fermentation samples

Ten ml of fermentation medium was centrifuged at 6000 rpm for 15 min. Then the pellets were suspended in 5 ml of sterile water and dried for 24 hrs at 100^0C. To cell suspension, 5 ml of Sodium Hypochlorite solution was added and incubated at 60^0C for 1 hour "[6]". The suspension was again centrifuged at 6000 rpm for 15 min and the supernatant was separated. To extract cell lipids and other molecules (except PHB) from supernatant, 5 ml of 96% (1:1 v/v) ethanol and acetone were added. Now 10 ml of chloroform was added to the tube by placing it in hot water bath (60^oC), to separate PHB from liquid broth. Chloroform was evaporated to obtain PHB crystals. To this 10 ml of 98% H_2SO_4 was added at 60^0C and kept for 1hr in water bath to convert PHB crystals into crotonoic acid. After cooling to 25^0C, the amount of PHB was determined spectrophotometrically at 235nm against H_2SO_4 as blank "[7]".

2.9 FTIR analysis of the polymer

The polymer extracted from the fermentation medium was analyzed qualitatively by FTIR to know the presence of different functional groups. 1mg extracted sample of PHB was dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film "[8]" and subjected to FTIR analysis by using FTIR spectrophotometer. Spectra were recorded in 4000 cm-1 to 400 cm- 1 range.

3 Results and Discussion

The ecological awareness on synthetic plastics impelled the development of new, eco friendly materials, especially for single use plastic items. Polyhydroxybutyrate (PHB) is natural thermoplastic polyester which is produced by bacterial fermentation and degrades fully in the environment without forming any toxic products. Polyhydroxybutyrate "[1], [9]"was found an alternative because it is easily biodegradable. The use of biodegradable plastic appears to be a promising solution to the problems arrived by the use of synthetic plastics.

Recent studies mainly concentrating on the PHB producing micro organisms "[8]" under the conditions of nutrient stress especially marine bacteria are more efficient in producing this type of unique compounds due to the versatile environment they survive. The parameters chosen for the research project work were mainly concentrating on the isolation of bioplastic producing organisms and the large amounts of PHB production by fermentation.

Table 1: The physico-chemical properties of collected marine water sample are listed below.

Physico chemical properties	Chirala Water sample
pH [a]	7.6
Temperature [b]	25^0 C
Turbidity [c]	<0.1 NTU
Salinity [d]	29.68 ppt
Electrical conductivity [e]	0.0447
Total dissolved solids(TDS) [f]	26%
BOD [g]	365 mg/L
COD [h]	950 mg/L
Dissolved oxygen content [i]	72%
Total nitrogen content [j]	10%
Total carbon content [k]	11.1%

- [a,b,c,e,f,j,k] Standard Operating Procedures for Water Sampling- Methods and Analysis", Dept.of Water, Govt of Western Australia, 2009
- [d,g,h,i] Practical Microbiology, Dubey and Maheswari , Third revised edition in 2012 published by S. Chand & Company LTD.

Three different strains were isolated from the marine water sample collected from chirala coastal area of Andhra Pradesh and named them based on the coastal area of marine sample from which the strains were isolated. This area has been selected as they have not been explored previously. Among them **CMM3 (**Fig 1) identified positive for presence of lipophilic PHB granules.

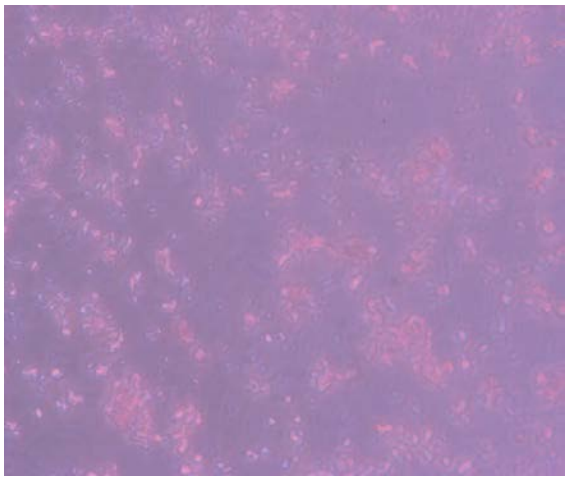

Fig 1: PHB granules of CMM3 under phase-contrast microscope

3.2 Morphology of CMM3:

At the early growth stages CMM3 colonies appear in ash colour and after 2 to 3 days colonies develop yellow colour mycelium (Fig 2). CMM3 was found to be gram positive curved rods with no specific spores (Fig 3).

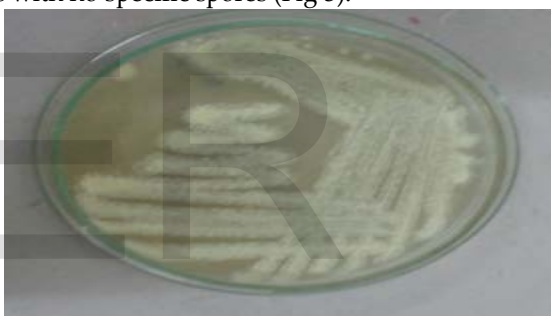

Fig 2: CMM3 colony morphology

Figure 3: Gram staining of CMM3

Table 2: Biochemical properties of CMM3

Name of the Test	CMM3
Indole Production Test	-ve
Methyl Red Test	-ve
Voges Proskauer Test	-ve
Citrate utilisation Test	-ve
Oxidase Test	-ve
Catalase Test	+ve
Urease Test	+ve
Carbohydrate Fermentation Test	+ve
Starch hydrolysis	+ve
Nitrate Reduction	+ve

Amplification of 16s r RNA and other genotypic approaches are taking over traditional ways for "[10]" identification of genus to the species level. Among the used methodologies PCR is widely practiced. The gene 16s rRNA is the tool mainly used for molecular identification of bacteria. The NCBI BLAST search program showed that the sequence data "[11]" of isolate CMM3, which showed positive response to PHB production was also subjected to 16s rRNA sequencing and identified as actinobacterial "[12]" member which has 97% homology with *Nocardiopsis potens*.

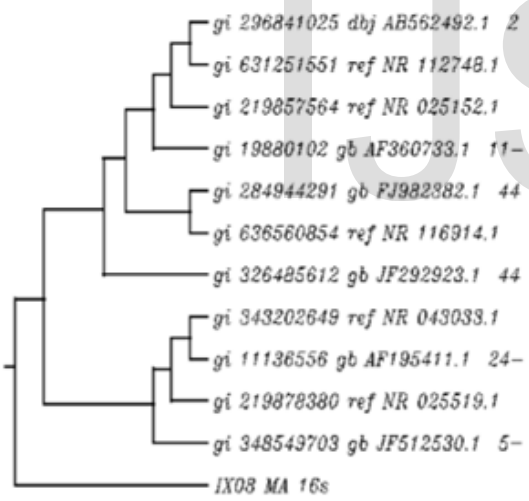

Figure 4: Phylogenetic tree of CMM3

During the optimization of cultural conditions the better yield obtained in N-limited medium supplemented with starch and yeast extract (2:0.2). while, Akanksha et al. "[3]" reported that maltose and yeast extract gives the better yield. The maximum yield of the product 36.2µg/ml arrived after optimizing theenvironmental conditions like pH and temperature of the fermentation medium. The production of polyhydroxybutyrate at various stages of optimization "[5]" is given in the following tables (3 to9).

Table 3: PHB production in media of various carbon sources

Carbon source (1%)	PHB production(µg/ml)
Control	0.8
Glucose	6.2
Sucrose	6.0
Maltose	6.4
Fructose	6.2
Starch	**7.0**

Table 4: PHB production in media of various nitrogen sources

Nitrogen source (1%)	PHB production(µg/ml)
Control	0.8
NH_4Cl	8.0
NH_4SO_4	7.2
NH_4NO_3	8.2
Yeast extract	**10.8**

Table 5: Effect of different concentrations of best N source on PHB production

Conc. Of N-source	PHB production(µg/ml)
Control	0.8
1:0.2	**17**
1:0.4	9
1:0.6	7
1:0.8	8.2
1:1	8.3

Table 6: Effect of different concentrations of best C source on PHB production

Conc. Of C-source	PHB production(µg/ml)
Control	0.8
1.0:0.2	8.2
1.5:0.2	8.6
2.0:0.2	**9.2**
2.5:0.2	9.0
3.0:0.2	7.9

Table 7: Effect of different NaCl concentrations on PHB production

NaCl concentration	PHB production(µg/ml)
Control	0.8
0.5	14.6
1.0	12.4
1.5	12.2
2.0	17.0
2.5	**20.0**

Table 8: Effect of pH on PHB production

pH	PHB production(µg/ml)
Control	0.8
5.0	7.0
6.0	**35.2**
7.0	24.8
8.0	13
9.0	9.0

Table 9: Effect of different Temperatures on PHB production

Temperature	PHB production(µg/ml)
Control	0.8
20^{0}C	8.0
30^{0}C	**36.2**
40^{0}C	18.8
50^{0}C	11.0

3.4 FTIR analysis

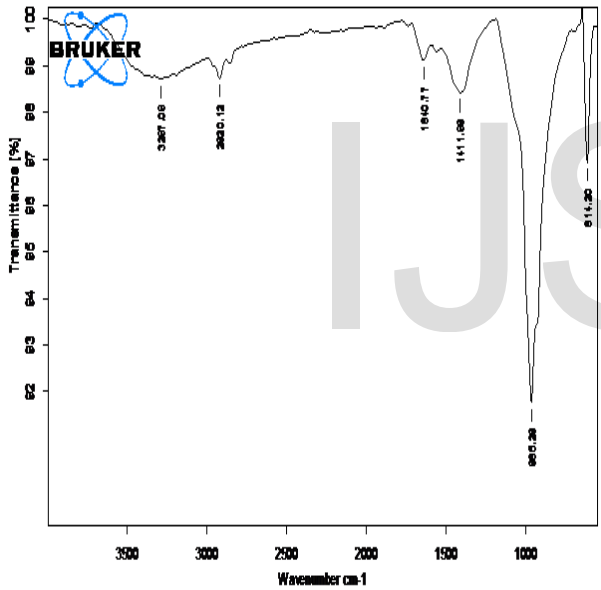

Figure 5: FTIR spectra of CMM3

1640 cm-1 (thioester C=O); 1411 cm-1 (CH, CH2,CH3); 965cm-1 (C-O-C)

FTIR spectra predicted the presence of PHB functional groups i.e., 1411cm^{-1} (CH, CH2, CH3); 1640 cm^{-1}(C=O); 965cm-1 (C-O-C). All absorptions due to the PHB moiety appeared in the spectrum and in a absorption band at 1640cm " was detected a thioester (C=O) bond "[8]".

4 Conclusions

Among the 3 isolates, CMM3 was identified positive for the production of PHB. CMM3 positive for Catalase, Urease, Carbohydrate fermentation, Starch hydrolysis and Nitrate reduction tests (Table 2) and negative for IMVIC and oxidase tests. CMM3 identified as actinobacterial member which has 97% homology with *Nacardiopsis potens* . Starch (2%), yeast extract (0.2%), C:N ratio (2:0.2), Nacl concentration (2.5%), pH(6.0) and temperature(30^{0}C) are optimal conditions for fermentative production of PHB by CMM3 which gives 36.2µg/ml All absorptions related to the PHB moiety appeared in the spectrum.

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