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Microbial synthesis of Bio-polymers of Polyhydroxybutyrate (PHB) from spent wash as low-cost carbon source in the medium

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ABSTRACT

The use of plastics is widespread, from the automotive to the medical industries. The plastic takes a very long time to break down and is bad for the environment. It is best to use biodegradable plastics rather than such non-biodegradable polymers. The biopolymer polyhydroxybutyrate (PHB) has characteristics similar to those of synthetic polymers and is vulnerable to microbial deterioration in the environment. Microorganisms like yeast and bacteria produce PHB when under stress. The primary objective of the study was to isolate bacterial strains from soil capable of generating PHB. Sudan Black B was used to detect PHB for the initial screening of the isolates. The method of digestion with sodium hypochlorite was used to extract PHB. PHBs that had been extracted were measured using UV-VIS spectroscopy and identified using FTIR and DSC.

Key word: *Biopolymer, PHB, Spentwash, FTIR, DSC*

Introduction

Research on biodegradable and biocompatible polymers has been inspired by the negative environmental effects of plastic disposal as well as the advancement of biotechnology (Lee *et al.,* 1994). The polyhydroxyalkanoates (PHA) produced by microbes have the greatest potential to make a significant contribution to the field of bioplastics. Many Gram-positive and Gram-negative bacteria have the ability to accumulate PHAs, a class of natural polyesters. The PHA discovered in bacteria with the greatest distribution and best characterization is polyhydroxybutyrate (PHB). Under conditions of nutritional stress, these polymers are stored intracellularly and serve as a carbon and energy reserve (Senior *et al.,* 1973).

This polymer's characteristics are comparable to those of synthetic or petrochemical-based polymers. According to Brandl *et al*., 2009, PHAs is biodegradable, water insoluble, non-toxic, biocompatible, piezoelectric, thermoplastic, and elastomeric. It is often used in numerous contexts including packaging materials, long-term drug dosages, medications, pesticides, fertilisers, the cosmetics industry and disposable items including razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, mugs, and so on.

The usage of PHAs as alternatives for the traditional synthetic polymeric material with a wide variety of applications have been compared with plastic because to the high cost of PHAs' manufacture. To lower the cost of producing biopolymer, it is necessary to use inexpensive carbon sources, nutritional supplements and feeding substrate techniques (Lee *et al*., 1999). Spent wash generated from distillery in-

[1]Research Scholar, [2]HOD and Dean)

dustry has been used as carbon source for the production of PHB. Utilizing this inexpensive carbon source we can reduce production costs improving market competitiveness.

The goal of the current research project was to create PHB bio-polymer using inexpensive substrate (carbon source) spentwash.

Materials and Methods

Sample collection

Soil samples were collected from the Karad region of Maharashtra. The collected samples were placed in plastic bags and maintained at 4 °C till further use.

Enrichment and Isolation of PHB producing bacteria from soil sample

Sample was enriched in mineral salt medium (MSM) medium containing 10% spent wash at 30^{0}C for 10 days on shaker. After enrichment, the various dilutions were plated on nutrient agar medium plates. During the 24-48-hour incubation period at 30 ^{0}C, isolated colonies were subcultured and maintained on nutrient agar slant at 4 ^{0}C.

Screening for microorganisms producing PHB

Using Sudan Black B dye, the synthesis of PHB was qualitatively detected in all of the bacterial isolates. Nutrient agar media was autoclaved, poured into petri plates, and allowed to solidify for screening of PHB producers. Bacterial isolates were inoculated into each of the eight equally sized parts of the plates. The plates were incubated for 24-48 hours at 30^{0}C. The colonies were covered with an ethanolic solution containing (0.02%) Sudan black, and the plates were left for 30 minutes (Burdon, 1946 and Cain, 1947). The colonies which developed dark blue colour were regarded as producing PHB. Further detection by fluorescent was observed using nile blue A plate assay method (Cain, 1947). The promising isolate was maintained at 4 ^{0}C.

Characterization of PHB producing isolates at the morphological, biochemical and molecular level

The isolate's morphology was identified using gram nature and colony characteristics. The isolate have further characterised using a different of biochemical tests such as catalase and amylase production, the MR-VP test and carbohydrates fermentations. Furthermore, 16S rRNA gene sequencing was done. The National Centre for Biotechnology Information's (NCBI) BLAST tools and nucleotide sequence homology were then used on the sequence to find the nearest species conceivable. The phylogenic tree was prepared using Mega X.

PHB production studies

The 100 ml production medium with spent wash was prepared (pH.7.0), autoclaved and used for PHB production by the promising isolates. The 10^8 CFU/ml isolate was inoculated in medium and incubated for 72h at 30^{o}C on shaker at 120rpm. Then the PHB produced was extracted using chloroform method and quantified using crotonic acid standard graph method.

Extraction and quantitative analysis of PHB

Sample was taken from production medium and centrifuged at 10,000 rpm for 20 min. to separate biomass. The supernatant was discarded and pellet was taken and dried. The pellet was resuspended in 4% sodium hypochlorite and incubated at 30 ^{0}C for 30 min. The mixture was then centrifuged at 4000 rpm for 10 min. to sediment lipid granules. The supernatant was discarded and the pellet was washed with acetone and ethanol. The pelleted granules were dissolved in hot chloroform in water bath and filtered. To filtrate, concentrated H_2SO_4 was added, which converted polymer to crotonic acid and absorbance was read at 235 nm against concentrated H_2SO_4 blank on UV-VIS spectrophotometer. Crotonic acid powder was used as standard. Standard graph of concentration of crotonic acid v/s absorbance was prepared.

Polymer Analysis

Fourier Transformed Infrared Spectroscopy (FTIR) analysis

FT-IR analysis of polymer samples was performed on FTIR spectrophotometer in the range 4000-600 cm, hired service from Yashawantrao Chavan Institute of Science, Satara, Maharashtra (India).

Differential Scanning colorimetry (DSC) analysis

Differential Scanning colorimetry (DSC) analysis was done on SDT Q600 V20.9 series instrument from Shivaji University, Kolhapur.

Results and Discussion

Isolation of PHB-producing bacteria

Total 75 isolates from various natural sources were obtained and cultured on nutrient agar medium. The simplest initial screening method for bacteria that produce PHB is Sudan black B staining. Sudan black B staining revealed dark spots inside the pink-coloured cells in the isolates were found to be positive for PHB granules using Nile Blue A, a more specialised dye for PHB granules. The most promising bacterial isolate was gram positive and spore-forming in nature, and it was used for production studies.

Identification of best promising Isolate

On the basis of its physical and biochemical characteristics, the isolate was identified as a Bacillus species. It had the closest match to the *Bacillus cereus* strain when isolate *Bacillus* was further detailed using 16S r-RNA genes for sequence homology using BLAST (Figure 1). The 16S r-RNA sequence of the isolate has been submitted to the NCBI gene bank (Accession No. OP430573).

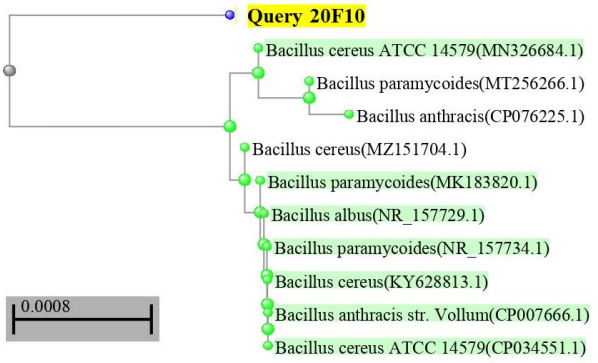

Fig. 1. Phylogeny of isolate 20

Extraction of PHB

The pellet of isolate when subjected to extract PHB with sodium hypochlorite and after drying it was observed to accumulate about 7.22% of PHB after72 h. PHB concentration from standard curve was 16. 25 µg/ml of extract by UV spectrophotometric method.

Fourier Transformed Infrared Spectroscopy (FTIR)

The figure 2 shows polymer made from *Bacillus subtilis* GP 20 isolate was used to record an IR spectra in the 4000-500 cm-1 region. Two prominent absorption bands at 1646.91 and 1083.80 cm-1, corresponding to the C=O and C-O stretching vibrations, are visible in the IR spectrum (Fig. 2). The methyl and methylene groups' C-H stretching vibrations cause the absorption bands at 2854.13 and 2925.48 cm-1. These exceptional absorption bands attest to the poly-hydroxybutyrate structure (Sharma, 2007)

Differential Scanning Colorimetry (DSC) analysis of PHB polymer

(In Fig.3) The TG DT curve, which is depicted in figure, measures the thermal stability and compositional changes related to the calcinations processes.

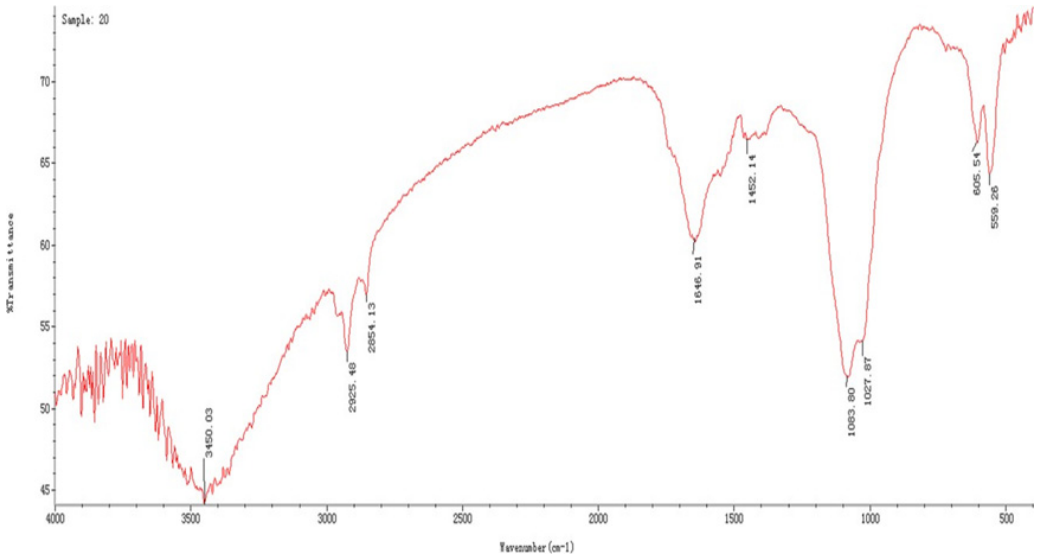

Fig. 2. FTIR analytical graph of isolate 20

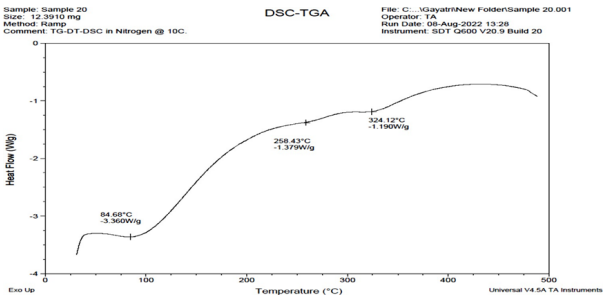

Fig. 3. DSC analysis of isolate 20

The TG DT curve exhibits a two-stage loss of weight at 84.68 °C and 258.43 °C, respectively. At a temperature of 84.68 °C, weight loss is thought to be mostly caused by the elimination of water molecules, but at a temperature of 258.43 °C, weight loss is thought to be primarily caused by the breakdown of the polymer chain of polyhydroxybutyrate macromolecules.

The best isolate used in this study was characterized morphologically and biochemically as shown in Table 1.

Characteristics	Observation
Morphological characteristics	Gram positive, spore forming rods
Colony characterization:	
Colour	white colour
Shape	Circular
Elevation	Convex
Biochemical characteristics:	
Glucose fermentation	Acid production
Lactose Fermentation	No acid production
MR test	Negative test
VP test	Positive test
Catalase production test	Positive test
Amylase production test	Positive test

Discussion

The isolate *Bacillus cereus* GP 20 was found to be potent producers of PHB with yield of 7.22%. Thus the isolate has dual potential of PHB production as well as using distillery spentwash.

Conclusion

The bacterial isolate i.e., *Bacillus cereus* GP20 can be used for PHB production using distillery effluent as substrate, by refining production conditions.

Conflict of interest

There is no conflict of interest among the author.
Ethics of human and animal experimentation
The authors ensure that the study does not involve any type of experiments on humans or animals.

Author's contribution

Gayatri V. Patil did all the laboratory work in the present study and prepared manuscript. Dr. Girish R. Pathade made the proof reading of the manuscript.

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