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ORIGINAL ARTICLE **OPEN ACCESS**

PRODUCTION AND CHARACTERIZATION OF POLYHYDROXYBUTRATE BY *BACILLUS SUBTILIS* GP 10 USING DISTILLERY WASTE AS A SUBSTRATE

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ABSTRACT
The plastics are used in almost every field of production, from automotive to medical. The plastic is harmful to the environment and takes a long time to degrade. Biodegradable plastics should be used to avoid the use of such non-biodegradable polymers. The polyhydroxybutyrate (PHB) is a type of biopolymer that resembles synthetic plastics in properties and is susceptible to degradation by environmental microorganisms. In stressful situations, microorganisms such as yeast and bacteria produce PHB. Isolation of PHB-producing bacterial strains from soil was the main aim of the research. Primary screening of isolates was performed using Sudan Black B for PHB production. Extraction of PHB was performed using the sodium hypochlorite digestion method. Extracted PHBs were quantified by UV-VIS spectroscopy and characterized by FTIR and DSC. The percentage and amount of PHB extracted were 25%.per biomass, respectively. We were able to extract significant amounts of PHB from Bacterial isolates. As a future perspective, improvements in PHB production can be made by using distillery spent wash as raw material.
Keyword: *Bioplastic, Bacillus subtilis, FTIR, DSC*

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INTRODUCTION

Plastic is made from a variety of chemicals, many of which are known to be highly poisonous and to constitute a severe threat to the biosphere. Along with having a negative impact on the environment, these compounds can also result in a variety of issues like cancer, brain system and immune system damage, birth and birth defects. The main component of plastic, polypropylene, is a petroleum-based material that is getting more and more expensive. Due to their high biodegradability, environmental friendliness, and sustainability, biosynthetic and biodegradable biopolymers have garnered a lot of attention from people who are concerned about the environment.

Under nutrient-limited conditions, a wide variety of bacteria synthesize PHBs (polyhydroxybutric acid) as intracellular carbon and energy reserve material [1]. Due to its physical similarities to the petroplastic polypropylene and advantage of being completely biodegradable (into water, carbon dioxide, and methane under anaerobic conditions) by microorganisms in natural environments, poly-hydroxybutyrate (PHB), the first identified polyhydroxyalkonate (PHA), is receiving a lot of attention [2]. By developing new strains, optimising the fermentation and separation procedures, and utilising cheap carbon sources, the high production cost of PHB can be reduced.

Out of all the key sugar manufacturing units, including the mill house, process house, boiler house, alcohol producing unit, and distilleries, the contribution from the sugar industries is worst in terms of effluent generation on an annual basis [3]. The biochemical oxygen demand (BOD) of the Distillery effluent is considerable, and if it is discharged without being treated, the increased BOD affects the aquatic ecology and ultimately has an influence on human health. To meet regulatory requirements, the Distillery industry must spend a significant amount of money treating waste water. Thus, by producing the bioplastic utilising this organic waste as a nutrition source, the two distinct problems, namely the pollution caused by synthetic plastic and the development of organic rich waste from sugar companies, may be uniquely handled.

However, given the amount of oxidizable carbon present, this carbohydrate waste lacks essential mineral nutrients, particularly nitrogen, and may still need nutrient supplementation. Here, an effort was made to

produce PHB using waste water from the Distillery industry after nutrient modification by using isolate *Bacillus subtilis* GP 10 (NCBI accession no. OP415431).

MATERIAL AND METHODS

Sample collection

Samples of soil were taken from the Karad region of Maharashtra. In order to preserve them for later processing, collected samples were put in plastics bags and kept at 4 °C.

Preprocessing of the substrate

The Jayawant Sugar industry, Dhawarwadi, karad, India provided the untreated distillery industry effluent, which was then kept there at 4 °C until it was needed. The unwanted solid components were filtered out of the wastewater using a muslin cloth. Waste water that had been diluted and filtered was utilised to create PHB.

Isolation of bacteria that produce PHB

One gram of soil was added in mineral salt broth(MSM) medium for enrichment (g/L of urea, yeast extract, KH_2PO_4, Na_2HPo_4, $MgSO_4$ $7H_20$, $CaCl_2$, and glucose 40) containing 10% spent wash and trace elements ($ZnSO_4$, $FeSO_4$, $(NH_4)_6MO_70_{24}$ $4H_2O$, and H_3BO_3) incubated at 30 ◦ C for a week and Nutrient agar was used for isolation of microorganisms. The plates were incubated at 30° for 48 h. After incubation, well isolated representative colonies were selected and used for screening.

Screening of PHB-producing strains

Isolated strains were subjected to primary screening by sudan black B slide and plate assay method [4]Sudan black B positive isolates were further secondarily screened with Nile blue A plate assay method on nutrient agar medium containing 0.0005 g Nile blue. The isolates which showed bright fluorescence on exposure with UV light were selected as PHB producers.[4,5] The best promising isolate was used further PHB production studies .

Molecular, Biochemical and Morphological Characterization

By using the common microbiological techniques, the isolate's morphology was recorded. A number of biochemical tests were used to characterize the isolate's including tests for catalase production, MR-VP test, enzymatic characteristics, and carbohydrates fermentations. Additionally, 16S rDNA was amplified for molecular characterization. The sequence was then subjected to a nucleotide sequence homology and BLAST tools available at the National Centre for Biotechnology Information to search for the closest possible species (NCBI). Mega X was used for phylogenic tree.

Extraction of PHB From microorganism

dPHB was extracted from isolates using the sodium hypochlorite extraction method . After culturing in E 2 production medium for 72 hours at 30°C, the cells were centrifuged at 10,000 rpm for 15 minutes. The pellet was suspended in 4% sodium hypochlorite solution and incubated for 30 min .at 30°C. The bacterial cells were fully lysed, leaving only the lipid inclusion bodies intact. After 10 min. standing, the mixture was centrifuged at 8000 rpm for 15 min, then the pellet containing PHB was washed successively with water, alcohol and acetone and dried at 30°C. The residual biomass was estimated as the difference between the weight of dry cells and the dry weight of PHB [6]. The percentage of PHB was calculated using the following formula:

%PHB = dry weight of extracted PHB x 100 / weight of dry cells

Polymer Analysis

Fourier Transformed Infrared Spectroscopy（FT-IR)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

DSC SDT Q600 V20.9 Build 20 instrument was used to perform thermal analysis of his PHB samples under flowing nitrogen atmosphere at a heating rate of 30°c C to 500°C min^{-1} . The α - Alumina powder was used as a reference material using an aluminum sample holder to record thermograms . Small samples (5-10 mg) were removed to ensure temperature uniformity and good reproducibility. Two runs were performed under the same experimental conditions to verify reproducibility.

RESULTS AND DISSCUTION

Isolation PHB-producing bacteria isolation

On nutrient agar medium, 75 isolates from various natural sources were Obtained. Sudan black B staining is the easiest first-line screening procedure for bacteria that produce PHB. Using Nile Blue A, a more specialised dye for PHB granules, strong orange colour fluorescence was seen under UV transIluminator in the isolates that were found positive for PHB granules following staining with Sudan black B (i.e.,

revealing dark spot inside the pink coloured cells)The best promising bacterial isolate gram positive, spore forming in nature which was used for production studies.

Identification of best promising Isolate

Table1: The best isolate used in this study was characterized morphologically and biochemically

	Observation
Morphological characterstics	Gram positive ,spore forming rods
Colony characterization	
Colour	Cream white colour
Shape	Circular
Elevation	Flat
Biochemical characteristics	
Glucose fermentation	+ve
Lactose Fermentation	-ve
MR test	+ ve
VP test	-ve
Catalase production test	+ve
Amylase production test	+ve

The isolate was recognised as a Bacillus species based on its morphological and biochemical properties. When isolate *Bacillus* was further described using 16S r-RNA genes for sequence homology using BLAST and it was referred to as *Bacillus subtilis* because it had the closest match to the *Bacillus subtilis (*Figure 1). The isolate's 16S r-RNA sequence has been uploaded to the NCBI gene bank (Accession no. OP415431).

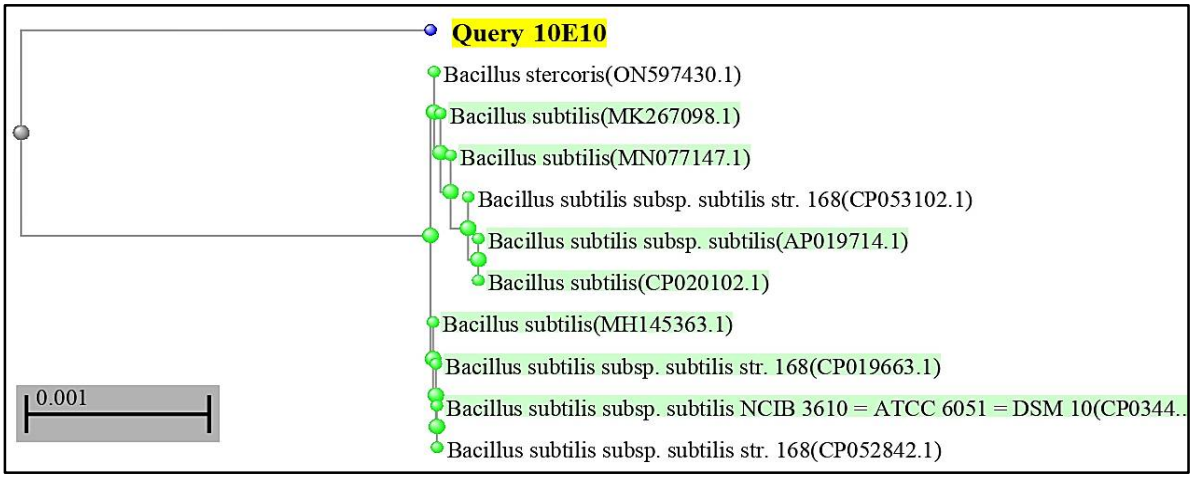

Fig 1. Phylogenetic tree of isolate no. 10

Extraction of PHB

The pellet of isolate when subjected to extract PHB with sodium hypochlorite and after drying it was observed t o accumulate about 25 % of PHB after 72 h.

Fourier Transformed Infrared Spectroscopy (FTIR).

(In figure 2) An IR spectrum in the range 4000-500 cm-1 was recorded using a polymer extracted from *Bacillus subtilis* GP 10 isolate . The IR spectrum (Fig. 3) shows two strong absorption bands at 1652.70 and 1078.01cm-1, characteristic of C=O and C-O stretching vibrations, respectively. The absorption bands at 2850.27 and 2962.13 cm-1 are due to C-H str etching vibrations of the methyl and methylene groups. These outstanding absorption bands verify the shape of poly-β-hydroxybutyrate.[7]

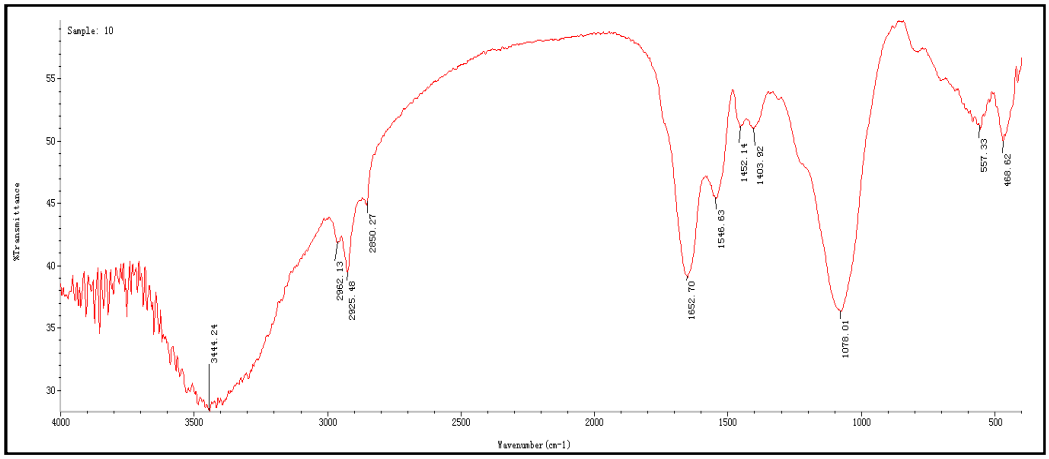

Fig 2. FTIR analysis of PHB of isolate 10

Differential Scanning Colorimetry (DSC) analysis of PHB polymer

(In Fig. 3) ,The TG DT curve measures the thermal stability and compositional changes connected with the calcinations processes and are illustrated in figure. TG DT curve shows weight loss in two steps at 75.12 ^{0} C and 324.12^{0} C respectively. It is consider that, major weight loss at 75.12 ^{0} C temperature is due to removal of water molecule [8] while weight loss at 324.12^{o} C is due to degradation of the polymer chain of biomolecules of polyhydroxybutyrate [9].

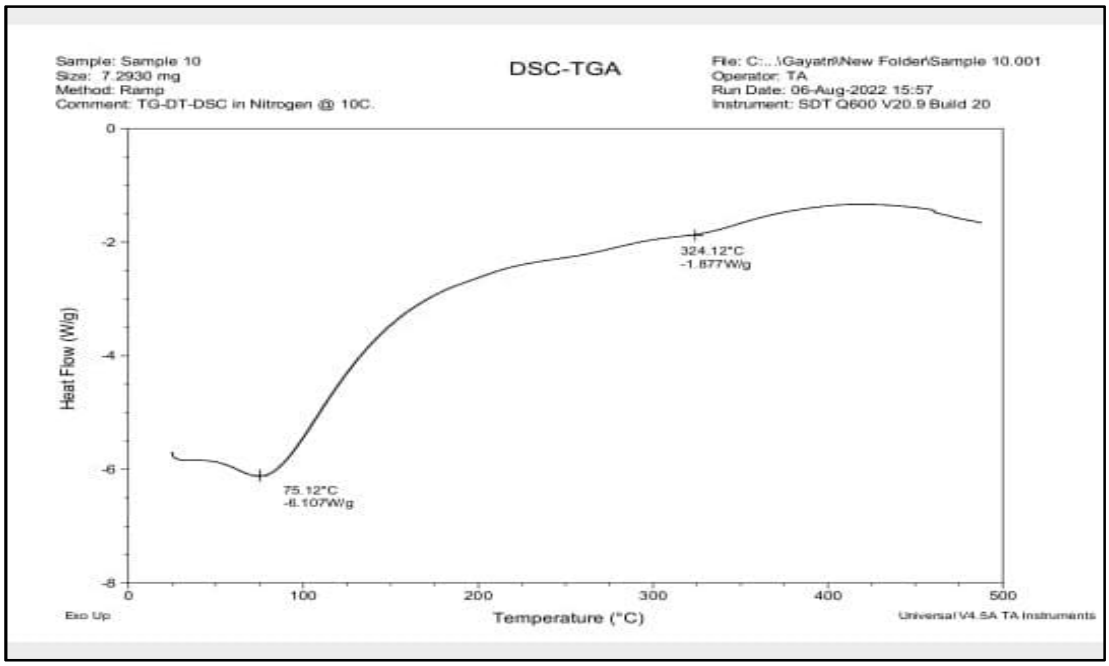

Fig 3. Thermogram by DSC analysis of PHB.

DISSCUSSION

The isolate *Bacillus subtilis* GP 10 was found to be potent producers of PHB yield 25%. Thus the isolate has dual potential of PHB production as well as significant degradation of distillery effluent.

CONCLUSION

The bacterial isolate i.e. *Bacillus subtilis* GP 10 can be used to commercially for PHB production using distiilery effluent as substrate ,by further optimization of 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.

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