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PRODUCTION, ISOLATION, SCREENING AND EXTRACTION OF POLYHYDROXYBUTYRATE (PHB) FROM *BACILLUS SPS* USING TREATED SEWAGE SAMPLE

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ABSTRACT

The amount of plastic waste increases every year and the exact time needed for its biodegradation is unknown. In the current scenario plastics and synthetic polymers are mainly produced using petrochemical materials that cannot be decomposed. PHBs are reserve polymers produced by a wide range of bacteria. They are accumulated as intracellular granules which are stored form of carbon and energy materials. Polyhydroxybutyrate (PHB) is fully biodegradable polyester with optical activity, thermoplastic and elastomeric features and has good barrier properties. The present study aims at the isolation and production of PHB from Bacillus sps form the treated sewage sample.PHB granules are identified by sudan black B staining and standard curve of PHB is studied by double beam UV-VIS Spectrophotometer. The medium used for the PHB isolation and production was simple and cost effective. Present investigation revealed the utilization of treated sewage sample for bioplastic production.

KEY WORDS

Polyhydroxyalkanoate, Polyhydroxybutyrate , Thermoplastic, Biodegradable.

INTRODUCTION

Plastic materials have become an integral part in our life as a basic need but they are causing serious Environmental problems due to their non biodegradability. They are widely applicable in packaging films, wrapping materials, shopping and garbage bags, clothing, fluid containers, toys, household, industrial products and building materials [1]. Synthetic polymers obtained from Petrochemicals causes air pollution only because they are not degradable in soil for long time. For this reason, a microbial plastic polyhydroxybutyrate (PHB) has gained importance because of its easily degradable nature. Environmental pressures are forcing on polymer manufactures to consider biodegradable polymers as an alternative polymeric material. PHB and polyhydorxyalkanoic acids, biodegradable thermoplastics can be produced from a wide range of substrates by using bacteria.[2]

Biodegradable polyesters are polyhydroxy alkonates (PHAs), polylactides, aliphatic polyesters, and polysaccharides. The PHA types are polyhydroxybutyrate (PHB), Polyhydroxyvalerate (PHV), polydroxyhexanote (PHH) and poylhydroxyoctanoate (PHO)[1]. Polyhydroxy alkonates (PHA) are polyesters of hydroxyalkanoates (HA) and consists of beta-hydroxyacyl as monomer [3].

PHB is an alternative source of plastics which has similar physical properties like polypropylene and

it can be easily biodegradable aerobically and anerobically [4].

PHB molecules are joined by ester bonds between the carboxyl and hydroxyl groups of adjacent molecules.PHB accumulates in distinct bodies, around 0.2 to 0.7 micrometers in diameter, that are readily stained with sudan black B for light microscopy and are seen as empty holes in the electron microscope. This is because PHB is hydrophobic, so it is dissolved by the solvents used to prepare specimens for electron microscope[5]. PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature in the range of 170-180^{o}C and a glass transition temperature in the range of 0-5 ^{o}C. Polyhydroxy butyrate (PHB) is an intracellular carbon and energy storage material synthesized by a great variety of bacteria. PHB was originally shown to be a constituent of lipid inclusions in the cells of Bacillus sps. (Winfred and Robards, 1973)[3].

At least 75 different genera of bacteria have been known to accumulate PHB as intracellular granules. Its production has most commonly been studied on microorganisms belonging to the genera Alcaligenes sps., Azotobacter sps., Bacillus sps., and Pseudomonas sps. [4]. Many researchers have explained that soil bacteria generally produce PHB. PHB production increases if ambient conditions like pH, temperature, nutrients are made available [6].

MATERIALS AND METHODS

(i) Sample collection and isolation of pure cultures:

Treated Sewage water sample was collected in sterile bottle from Sewage Treatment Plant, Pragathi Nagar , Kukatpally, Hyderabad .

1 ml water sample is dispensed in 9ml of sterile distilled water. This is mixed vigorously and 0.5ml from this is taken and added to another tube with 4 ml sterile distilled water to get a dilution of 10-1. This serial dilution is repeated to get dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at 37 ^{0}C for 48 hours. Colonies with different characteristic features were obtained and a single colony was picked, maintained as pure cultures on nutrient agar slants and stored at 4°C [7].

(ii)Screening of PHB producing Isolates by Sudan Black B staining:

Prepare thin smear on microscope slide and thoroughly air dry. Stain with Sudan black B (Schlegel et al., 1970) solution and let it stand for 10-15 minutes. Add more stain if the slide starts to dry out. Wash the slide with distilled water and counter stain with safranin for 5 minutes. Wash with distilled water and blot dry with tissue paper. Examine the slide under oil immersion microscope at 1000x magnification for PHB granules. Organism shows positive in blue violet (Aneja, 2001) and shows negative in yellow-brown [8 12].

(iii) Characterization of PHB producing organism

PHB producing organism was identified and characterized by morphological characters and biochemical tests

(a) Morphological characterization:

To study the morphological features the pure culture was spread on nutrient agar media plates and incubated for 24 hour at 37^{0}C and Colonies were observed [9]. Gram nature was studied by performing gram staining to the fresh 24 hour old culture [10].

(b) Biochemical characterization:

Different Biochemical tests were carried out for the isolated pure culture, which includes indole,

methyl red, voges-proskauer , citrate, urease and oxidase [9].

(iv)Production media:

A simple and economical nutrient broth medium was used for production of PHB by the organism. Four sets of 500ml of nutrient broth was prepared in 1000ml flask and sterilized at 121°C, 15lbs for 15 minutes. 2ml of fresh inoculum was added to each flask to carry out fermentation. The flasks were incubated at 37°C for 48hrs in rotary incubator at 1000rpm. Samples were collected at an interval of every 4 hours to check the PHB production, but production of PHB was observed in the sample collected after 24 hours. (Prasana et al)

(v)Extraction of PHB:

After 48 hours of incubation at 37^{0}C, culture was collected and centrifuged at 10,000 rpm for 15min and add acetone and centrifuged for 10 min, and add alcohol and centrifuged for 10min. The pellet was digested with sodium hypochlorite solution (Chang et al., 1994) at 37^{0}C for 3 hrs. Then pellet was collected after centrifugation at 10,000 rpm for 10min, again centrifuge with acetone, and ethanol respectively for 5min at 10,000rpm. Finally pellet was introduced to hot chloroform (Sing and Parmar, 2011) and kept for incubation at 60^{0}c for 10min[11 13]. Shake well and filter the entire solution by using filter paper. Extracted PHB was estimated using Double Beam UV-VIS spectrophotometer (Systronics) (Vidya.P.Kodali et al).

(vi) Estimation of PHB:

Take 15 mg of sample in to a clean test tube and add 10 ml of concentrated sulphuric acid , incubate in a hot water bath for 10 min at 60^{0}C . After 10 min read the absorbance at a range of 220 – 300 nm through Double beam UV-VIS spectrophotometer (Systronics). Concentrated sulphuric acid is used as the blank to measure the absorbance of the sample. (Adwitiya et al)

RESULTS

1. Morphological characteristics:

Isolated pure colonies of the culture were spread on nutrient agar plates and showed the following colony features and gram's reaction. The colonies isolated were identified as Bacillus sps. by observing their morphological features and gram nature .Refer result pictures Figure1, Figure2.

Table 1: Morphological characteristics of Bacillus sps.

Colony Features	**Result**
Shape	Irregular
Size	Medium-Large
Texture	Mucoid
Colour	Cream
Elevation	Flat
Density	Opaque
Margins	Irregular
Gram's Reaction	
Gram nature	Positive
Shape	Rods
Size	Short
Arrangement	Chains/Pairs

Nutrient Agar Plate

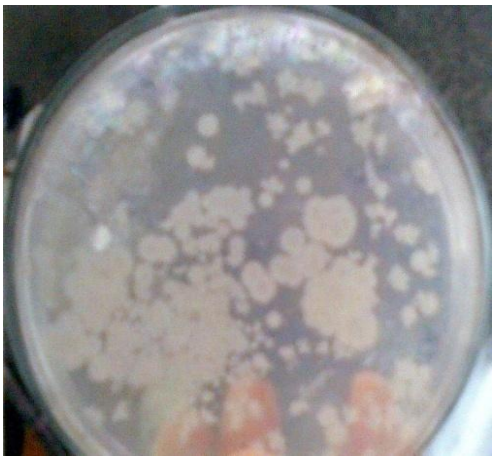


Figure 1 Nutrient agar plate with Bacillus sps colonies

Gram Staining

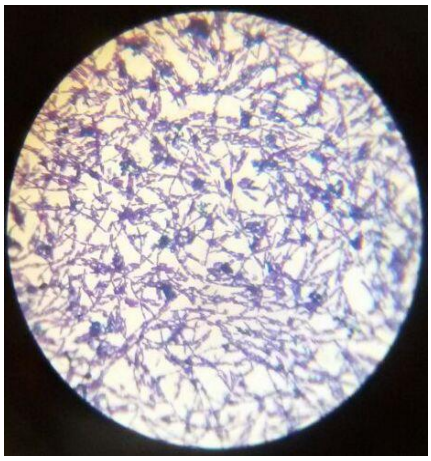


Figure 2 Microscopic field view of the gram staining of Bacillus sps under oil immersion

2. Biochemical tests for PHB producing Bacillus sps. :

The isolate was subjected to partial identification based on various biochemical tests according to Bergy's manual of systematic bacteriology. The isolate was confirmed as Bacillus sps. Which showed citrate, urease, catalase as positive and indole, voges proskauer, methyl red, oxidase as negative.

Table 2: Biochemical tests for PHB producing Bacillus sps.

Biochemical Tests	Results
Indole	-
Voges Proskauer	-
Methyl Red	-
Citrate	+
Urease	+
Catalase	+
Oxidase	-

3. Staining of PHB Granules:
Isolated organism was observed under oil immersion lens of light microscope and showed positive with Sudan black B staining. Poly hydroxyl butyrate granules appeared as clear blue- black dots with pink colour bacterial cells.

Sudan black B staining

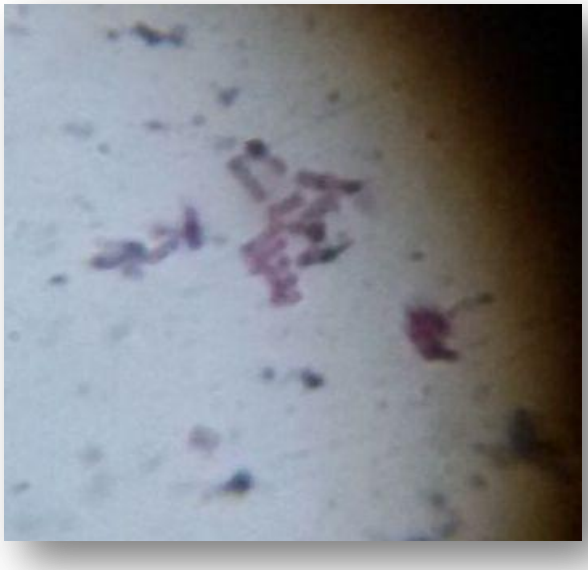
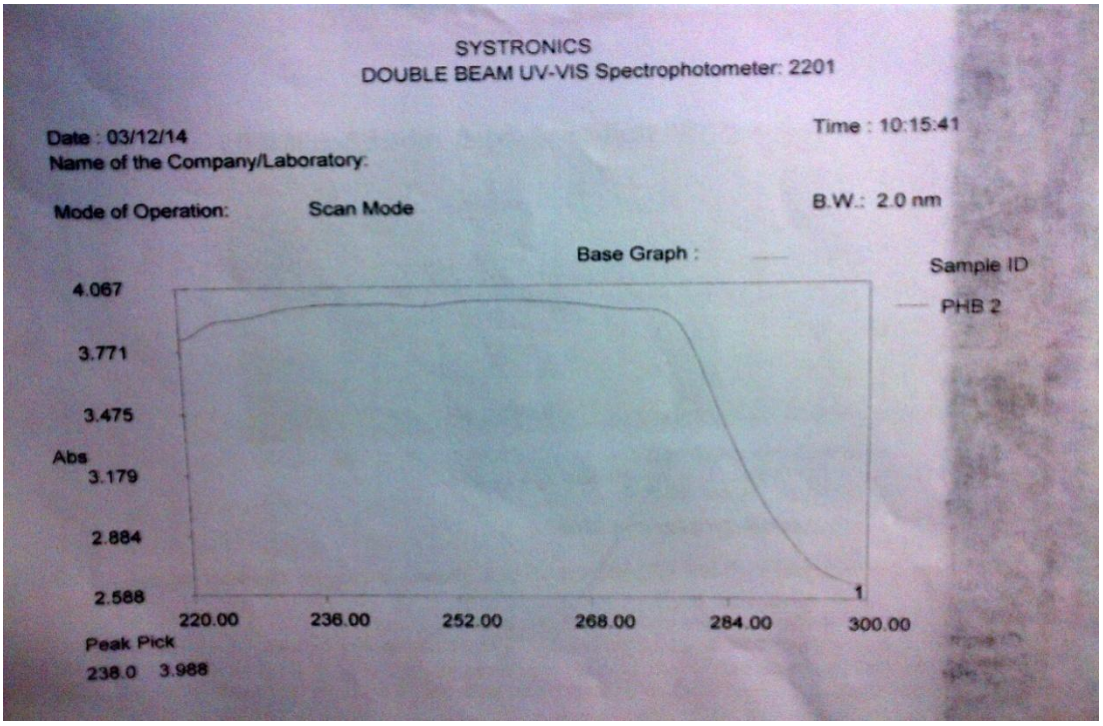


Figure 3: Microscopic field view of sudan black B staining of Bacillus sps showing blue-black PHB granules

4. Estimation of PHB from the isolated sample:
The production media by successive treatment of the solvents yields PHB in the form of pellet. Standard curve of PHB is studied by using the pellet. The extracted polymer from the *Bacillus* sps. as pellet which is solubilised in concentrated sulphuric acid. The absorbance of PHB was measured by double beam UV-VIS spectrophotometer with a band range of 220nm-300nm, Using concentrated sulphuric acid as blank. PHB peak of absorbance was observed at 238 nm. According to the standard PHB curve peak is observed at 235 nm.

Graph 1: Double beam UV-VIS spectrophotometer



Double beam UV-VIS spectrophotometer graph showing the peak point of PHB absorbents of the isolate at 238nm.

CONCLUSIONS

The main objective of sewage treatment is to produce disposable effluent without causing harm to the surrounding environment and prevent pollution. In the present investigation treated sewage sample is used for PHB production and *Bacillus* sps. was isolated from the sample. PHB which is a biopolymer produced from microorganisms for developing biodegradable plastics as an alternative solution for conventional chemically synthesised plastics. Production of biocompatible plastics is in demand in the world. *Bacillus sps* resulted as potential PHB producing organism by showing positive with Sudan black B staining and UV-VIS absorbance peak reading at 238nm using concentrated sulphuric acid as blank with respect to the standard PHB curve reading at 230 nm. Hence the proper disposal of treated sewage sample can be done by supplying to industries for bioplastic synthesis. Where the media and techniques used are simple and cost effective.

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