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Identification and Characterization of Polyhydroxybutyrate producing Paenibacillus sp.

KEYWORDS

Biopolymer, Polyhydroxybutyrate, Paenibacillus sp.

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ABSTRACT *Polyhydroxybutyrate producing bacteria were isolated from rhizospheric soil samples and were screened for PHB production by Sudan Black B followed by Nile Blue A staining procedures. The polymer production by the isolates was found to vary from 0.007- 0.129 g/L of PHB. The highest PHB yield was observed in R15, which was characterized for its morphological and biochemical properties. Based on its 16S rRNA gene sequences, isolate was identified as Paenibacillus sp. The ability of the organism to produce protease and amylase indicated possibility of using various wastes as carbon substrates for cost-effective PHB production. Paenibacillus sp.also showed resistance to antibiotics and heavy metals.*

INTRODUCTION

The growing reliance on petrochemical based plastics has significantly impacted our environment. The durability and strength that make these materials so useful also ensure their persistence in the environment complicating their disposal (Kalaivani and Sukumaran, 2013). Current worldwide dependence on fossil fuels for plastic manufacture, the scarcity of land for disposal and growing environmental concerns have fuelled research towards the development of eco-friendly biopolymers like bacterial polyhydroxyalkanoates (PHAs) (Godbole, 2016). PHAs are polyesters of various hydroxyalkanoates, synthesized by many bacteria having dual function as a reserve compound and as a metabolite accumulating in response to nutritional stress due to exhaustion of a single nutrient such as nitrogen, sulphur, iron, magnesium or oxygen (Shi et al., 2007). The biodegradability, compostability and piezoelectricity of polyhydroxyalkanoates deserve their current exploitation in industrial applications in packaging, medicine, agriculture, food industry etc. (Gumel et al., 2012).

Although more than 300 different microorganisms synthesize PHAs, few microorganisms such as Cupriavidus necator, Alcaligenes latus, Azotobacter vinelandii, Pseudomonas oleovorans and recombinant E. coli, have been studied for large-scale production. However, despite several advantages of PHAs, their commercialization has been met with limited success (Chanprateep, 2010). The PHA content and its composition are influenced mainly by the strain of the microorganism, the type and concentration of substrate and other growth conditions (Valappil, 2007).

The success of PHA production largely depends on the isolation of potent PHA producing bacteria and optimizing culture conditions (Nehra et al., 2015). Hence, present work was initiated to screen, isolate, identify and characterize potent PHB producing bacterial isolate from soil.

MATERIALS AND METHOD

Sample collection

Isolation of bacteria was performed by serial dilution technique onto nutrient agar, minimal medium and Ashby's Mannitol Agar using rhizosphere soil samples, from 3.0 to 4.0 cm below the surface. The plates were incubated at 29 ± 1°C for 24 – 72 h. Well isolated colonies of different morphologies were purified by subculturing 3-4 times on nutrient agar plates and then stored at 4°C till further use (Gupta et al., 2011).

Primary screening for PHB production

Primary screening of all the isolates for PHB production was carried out using Sudan Black B dye. Pure 18h old isolates were spot inoculated on PHA detection agar) [(g/L: Glucose 20, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 13.3, $MgSO_4$ 1.3, Citric Acid 1.7, Trace element solution 10 mL, [(g/L): $FeSO_4.7H_2O$ 10, $ZnSO_4.7H_2O$ 2.25, $CuSO_4.5H_2O$ 1, $MnSO_4.5H_2O$ 0.5, $CaCl_2.2H_2O$ 2.0, $Na_2B_4O_7.10H_2O$ 0.23, $(NH_4)_6Mo_7O_{24}$ 0.1, 35% HCl 10 mL]; Agar, 20.0 g/L pH 6.8 - 7.0) (Naheed et al., 2011) and incubated at 29 ± 1°C for 48 h. Ethanolic solution of (0.05%) Sudan Black B was poured over the colonies and the plates were kept undisturbed for 30 min followed by washing with ethanol (98 %) to remove the excess stain. The dark blue coloured colonies were taken as positive for PHB production (Phanase et al., 2011).

Secondary screening for PHB production

Sudan black B positive isolates were tested for PHB production by a more specific stain, Nile blue A. Isolates (18h) were grown on PHA detection agar containing 2% glucose and 0.5 g/mL of dye for 24-48h. The isolates showing bright orange fluorescence on irradiation with UV light were selected as PHB accumulators (Bhuwal et al., 2013).

Estimation of PHB produced by the isolates

Putative PHB producers screened on the basis of staining were subjected to production and quantification of the PHB under monophasic cultivation. PHB production was studied in 250 mL Erlenmeyer flasks containing 50 mL of sterile mineral medium [(g/L D/W): Glucose 20.0, $Na_2HPO_4.12H2O$ 4.8; KH_2PO_4 2.65; $(NH_4)_2SO_4$ 4; $MgSO_4.7H_2O$ 0.3; $FeSO_4.7H_2O$ 0.01; $CaCl_2.2H_2O$ 0.01 and 1mL trace element solution (g/LD/W): H_3BO_3 0.3; $CoCl_2.6H_2O$ 0.2; $ZnSO_4.7H_2O$ 0.03; $MnCl_2.4H_2O$ 0.03; $NaMoO_4.2H_2O$ 0.03; $NiCl_2.6H_2O$ 0.02 and $CuSO_4.5H_2O$ 0.01, pH 7.0] as production medium. They were inoculated with 1.0% (v/v) inoculum of 18h old culture grown on nutrient agar at 29 ± 1°C. After 72 h of incubation on rotating orbital shaker (Labtop Quality Lab Equipment, India) at 150 rpm at 29 ± 1°Cfor 72 h, medium was centrifuged for 15 min at 6000 rpm. The total cell dry weight (CDW g/L) was determined after drying the biomass a constant weight at 60°C for 24 h. The dried cells were held in 5

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volumes of chloroform and left overnight at room temperature. PHB crystals were obtained after evaporation of chloroform and used for gravimetric estimation (Lakshman, 2004).

Percentage of PHA was calculated as follows:

$$\text{Percentage of PHB} = \frac{Dry\ weight\ of\ extracted\ PHB\ (g/L)}{CDW\ (g/L)} * 100$$

The isolate producing maximum quantity of PHB was chosen for further work.

Molecular identification of isolate

After subjecting the isolate to morphological, cultural and standard biochemical tests according to Bergey's Manual of Systematic Bacteriology, (Sneath et al., 1986) the selected isolate was identified by molecular techniques. DNA extraction, 16s rRNA- PCR amplification and Sequencing was done SciGenom Labs Pvt Ltd., Cochin. 16S region was PCR amplified with 16sF and 16sR primers (16S-F:5'C-CGAA-TTCG-TCGA-CAACAGAGTTTGATCCTGGCTCAG-3', 16SR: 5'CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3') after isolating DNA. Amplicon was electrophoresed in a 1% Agarose gel and visualized under UV. Concentration of the amplicon was checked in a Nanodrop ND 2000. The amplicon purified using PureLink purification column (Invitrogen) was subjected tosequencing with forward and reverse primers in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequence analysis was carried out using bioinformatics tool BLAST of NCBI. Based on maximum identity score first few sequences were selected and aligned using multiple sequence alignment software ClustalW2. Dendrogram was constructed.

Study of hydrolytic enzyme activities

The isolate was evaluated for its ability to produce hydrolytic enzymes like protease, amylase and lipase in order to explore its ability to use waste materials as sole source of carbon. Mineral salts media agar plates containing 1% w/v of skimmed milk, starch and olive oil as the sole sources of carbon were used for detecting protease, amylase and lipase activity respectively. Enzyme activity was detected by spot inoculating 18h old isolate on respective plates incubated at 29 ± 1°C for 24h for detection of amylase and protease activity; and at 37°C for 7 days for lipase activity. A clear zone of hydrolysis observed around the bacterial colony was taken as an indication of production of exoenzymes (Israni and Srividya, 2013).

Detection of heavy metal resistance by the isolate

Minimum Inhibitory Concentrations (MIC) for heavy metals like mercury ($HgCl_2$), chromium ($K_2Cr_2O_7$), lead ($PbSO_4.2H_2O$) and copper ($CuCl_2$) was determined by supplementing nutrient agar plates with different concentrations of the metal salts (25 - 500µg/ml). MIC was taken as that highest concentration of the metal supporting the growth of the exponentially growing isolate after 48 h (Fleck et al., 2000).

Detection of antibiotic resistance by the isolate

Antibiotic Sensitivity Testing (AST) of the isolates was performed using standard Kirby Bauer disc diffusion method on Mueller Hinton agar by using commercially available antibiotic hexadiscs of Ampicillin (25µg), Norfloxacin (10µg), Co-Trimoxazole (25µg), Cephalothin (30µg), Nitrofurantoin (300µg) and Nalidixic acid(30µg) (HiMedia Laboratories, Mumbai). Hexadiscs were placed on Mueller Hinton agar plates swabbed with exponentially growing isolate. Zones of inhibition were measured and resistance pattern was recordedafter 24 h (Bauer et al., 1966).

RESULTS AND DISCUSSION

The rhizospheric soil layer associated with plants has been found to be colonized by bacteria potentially able to accumulate polyhydroxybutyrate as energy and carbon sources, thus, making it a good source for the isolation of PHB producers (Nehra et al., 2015). In the present work, 95 bacterial isolates of varying colony morphologies were picked-up randomly for further analysis from 12 different rhizospheric soil samples. Initial selection of 68 isolates was based on appearance of deep stained colonies after addition of Sudan Black B, followed by screening using Nile blue A. It is a rapid and sensitive, viable colony method allowing discrimination between PHA-negative and PHA-positive strains. Out of 95 isolates, 44 isolates (46.31%) appeared to be PHB producers showing fluorescence of varying degree. The putative PHB producers were evaluated for PHB production in shake flasks. The PHB yield varied between 0.007- 0.129 g/L. Isolate R15 produced 0.129 g/L PHB and hence was chosen for further study.

The isolate was identified using a series of biochemical tests and morphological characteristics. Rod-shaped Gram positive bacillus, producing ellipsoidal endospores in swollen sporangia in the sub terminal or terminal region of the cell produced off white, circular, smooth, large colonies. It produced acid from glucose, galactose, arabinose, xylose, lactose, mannitol, maltose, sucrose. The complete sequences were aligned to the homologous sequence available for Paenibacillus strains. The BLAST search using the sequences showed 97% homology to other GenBank sequences. A phylogenetic tree demonstrated that the isolate was the member of genus Paenibacillus. Sequence similarity calculations after neighbor joining analysis showed strong homology with other Paenibacillus strains available in the database.

Fig. 1: Phylogenetic relationship between isolate R15 and other selected Paenibacillus strains available is the database

Paenibacillusis a genus of facultative anaerobic, endospore forming bacteria, originally included in the genus Bacillus, and then reclassified. Hungund et al. (2013) reported PHB production (0.93 g/L) by *Paenibacillus durus BV-1after* 72 h of growth.

Utilization of a broad range of agroindustrial wastes as carbon feedstock for PHA biosynthesis can reduce production costs by 50% while overcoming disposal problems. Evaluation of *Paenibacillus* sp. revealed its ability to produce protease and amylase indicating possibility of using various food wastes. Resistance of the isolate to all the antibiotics tested and tolerance to 25 µg/mL of cobalt, 200 µg/mL of lead and 25 µg/mL of zinc showed its potential to utilize domestic waste water as substrate for PHA production.

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Volume : 6 | Issue : 11 | November 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

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