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ISOLATION, SCREENING, CHARACTERIZATION AND DETECTION OF MULTIDRUG RESISTANCE IN POLYHYDROXY BUTYRATE PRODUCERS FROM DIFFERENT SOIL SAMPLES

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ABSTRACT
Rapid accumulation of plastics in the environment affects wildlife, wildlife habitat and humans. Most of the animals can also be affected through entanglement, direct ingestion of plastic waste, or through exposure to chemicals within plastics that cause metabolic imbalances leading to metabolic changes. Hence, there is an urgent need for "eco-friendly" plastics. PHBs (Polyhydroxy butyrates) are good alternatives of synthetic plastics since they closely resemble synthetic plastics. Polyhydroxybutyrates (PHB) are biodegradable polyesters synthesized by many bacteria. Such bacteria were isolated and screened for PHB production by sudan black dye method. Also the antimicrobial activity of PHB producers was screened and the antibiogram was obtained. It was observed that among the bacterial isolates from three soil samples used in the present study, isolate 3 obtained from corn soil was able to produce maximum yield of PHB. Antibiotic profiling revealed a variation in the resistance pattern to the selected antibiotics. Among the three PHB producing bacteria isolated from soil, one isolate was found to be promising in PHB production and was also found to be multi drug resistant.

KEYWORDS: Polyhydroxy Butyrate, Sudan Black, Antibiotic, Biodegradable.

INTRODUCTION
Environmental pollution is a major problem in today's world. Pollutions may lead to critical problems in the global geochemical cycles as well as the sustainable habitation of humans as well as other organisms. All living forms suffer from the adverse effects of pollution, where, the main culprit is human, whose activities disturb the living systems along with many adverse changes in the environment (Kampa M. and Castanas E, 2008). Accumulation of non-degradable plastic bags in the environment is one of the major causes of pollution. Thousands of marine animals and more than 1 million birds die each year as a result of plastic pollution. Plastic accounts for approximately 10% of solid waste (Heap B, 2009) and contributes 80% of the wastes accumulating on ocean surface, land, shorelines etc. (Barnes DKA, 2009). Plastic bags have impact on atmosphere as well as on biodiversity. Plastic bags are from the same source as all plastic, fossil fuel. Like everything else manufactured from this non-renewable resource, it has two major drawbacks: manufacturing it emits considerable amounts of pollution, and the product is not biodegradable. Bio-plastics are bio-based, biodegradable plastics with almost similar properties to synthetic plastics. Bioplastics are made from variety of sources like polysaccharides, lipids and also proteins (Averous L, 2004; Hernandez-Izquierdo VM, Krochta JM, 2008; Siracusa V, 2008; Gonzalez-Gutierrez J, 2010). Production of Bio-plastics in large scale has become expensive and this has discouraged many. Hence, cheaper alternate sources are sought after. Biodegradable plastics are environment friendly and can replace all plastics products available at this time. Production of bioplastics will definitely result in reduction in emission of CO2 compared to traditional plastics. Poly-3- hydroxy butyrate (PHB) is 100% biodegradable and it is produced from various renewable sources (Godbole S, 2003). It has similar physical properties with polypropylene. A good reason for gaining priority is that use of these biodegradable and bio-based plastics will definitely reduce the pollution caused by CO2 emission from plastic wastes (Numata K, Doi Y, 2012). Several materials such as polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, polyisoprenoids and polyphenols are potential candidates for substitution of synthetic plastics(Steinbuchel A, 2001). Among these, polyhydroxyalkanoate (PHA), which belongs to the group of polyoxoesters has received intensive attention because it possesses biodegradable thermoplastic properties (Albuquerque MGE, 2007). PHA is synthesized by bacteria under unbalanced growth conditions. Some bacteria have been reported capable to

produce PHA as much as 90% (w/w) of dry cells during depletion of essential nutrients such as nitrogen, phosphorus or magnesium (Madison LL, 1999). PHA acts as an ideal storage compound due to its insolubility inside bacterial cytoplasm (Anderson AJ, 1990) and it was shown that the bacteria containing PHA storage materials would be able to survive during starvation period compared to those without PHA (Macrae RM, 1958). Gram positive bacteria is found to be the better producer of PHB because the outer membrane present in gram negative bacteria (*Cupriavidas necator, Alcaligenes latus*) contains LPS endotoxins that may induce a strong immunogenic reaction which limits its biomedical application. On the other, Gram positive bacteria lack LPS and they secrete protein at higher concentration and the potential use of cheaper raw materials made it a better source of PHA (Bhairavi ghate, 2011).

MATERIALS AND METHODS

Sample Collection and Isolation of Pure Cultures

Three different soil samples from rose garden, pumpkin and corn fields were collected in clean bags. One gram of soil sample was dispensed in 10ml of sterile distilled water. This was mixed vigorously and 1ml from this was taken and added to another tube with 9ml sterile distilled water to get a dilution of 10-1. This serial dilution was repeated to get dilutions of 10-2, 10-3, 10-4, 10-5, 10-6and 10-7. 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 C for 48 hours. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4°C.

Screening for PHB producing bacteria

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black Dye (Juan ML, 1998). For this screening of PHB producers, nutrient agar media supplied with 1% of glucose was autoclaved at 1210c for 20 min at 15lbs pressure. This media was poured into sterile Petri plates and allowed for solidification. The plates were divided into 5 equal parts and bacterial isolates were spotted. These plates were incubated for 24 hours. Now ethanolic solution of 0.02% Sudan Black B was spread over the Petri plates containing colonies and was kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from colonies.

Gram staining

Twenty four hours old culture was gram stained and the slide was observed under microscope for gram reaction. IMViC, oxidase and peroxidase tests were also done

Sudan black staining

Staining of cells with Sudan black B smears of cells deposited on a glass slide were heat fixed and stained with a 3% (w/v in 70% ethanol) solution of Sudan Black B (Sigma) for 10 min, followed by immersion of the slide in xylene until it was completely decolorized. The sample was counter stained with safranin (Sigma 5% w/v in deionized water) for 10 s, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by contrast microscopy. In this staining, lipid inclusion granules are stained blue black or blue grey, while the bacterial cytoplasm is stained light pink.

Identification of PHB Producing Isolates

PHB producing strains were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Systematic Bacteriology.

Morphological Characterization

Morphological features were identified by growing the cultures on nutrient agar media and gram staining was performed.

Biochemical Characterization

Different Biochemical tests were carried out includes, Catalase test, Oxidase test, Pigment production.

Cell Dry Weight

After 48hrs incubation at 37C, culture medium was collected and centrifuged at 10,000 rpm for 15min. Supernatant was discarded and the cell pellet was washed twice in deionized water, recovered (for 4 min at 10000 rpm at 4C). The cell pellet was dried 24 hr at 100oC then the total bacterial cell dry weight was determined as g/L.

Disruption of cells by chemical methods and PHB estimation: Nutrient broth was prepared in test tubes and inoculated with the isolates. The medium was incubated at room temperature for 24-96 hours. PHB was estimated at every 24 hours interval. About 5 ml of culture was taken and centrifuged at 10,000rpm for 10 minutes. Supernatant was discarded and the pellet was suspended in 2.5 ml of sodium hypochlorite and 2.5 ml of chloroform and it was incubated at 30ºC for 1 hour. The above content was centrifuged at 1500 rpm for 10 minutes at room temperature. The upper hypochlorite phase, the middle chloroform containing undisturbed cells and the bottom chloroform phase with PHB were obtained. The upper and middle phases were separated the contents were again centrifuged at 1500 rpm for 10 minutes at room temperature and the phase other than chloroform with PHB was removed carefully. Concentrated sulphuric acid was added to the chloroform phase containing PHB. It was then boiled at 100ºC in a water bath for 10 minutes. The absorbance of the samples was read at 230 nm using UV Spectrophotometer. The readings were plotted in standard graph of crotonic acid and the concentration of PHB was determined.

Crotonic acid standard
Since PHB is converted to crotonic acid on heating with concentrated sulphuric acid, pure crotonic acid salt was used as a standard for estimation. Crotonic acid was taken in concentrations from 1-10 µg/ml and to this 5 ml of concentrated sulphuric acid was added. The tubes were heated at 100ºC in a water bath for 10 minutes. The tubes were then cooled at 25ºC and the absorbance was read at 230 nm and a standard graph was plotted.

Estimation of PHB from the tested isolates
The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100 / DCW (g/L)

Antibacterial activity
Disc diffusion Test

The antibacterial activity of the selected antibiotics was determined by the disc diffusion method (Bauer, 1966). The zones of inhibition were calculated by measuring the diameters of the zone around the disc.

Determination of Minimal Inhibitory Concentration (MIC)
Two selected antibiotics were subjected for determining the minimal inhibitory concentration (MIC) by broth dilution method. The minimum inhibitory concentration of the antibiotics was estimated for the test isolates in triplicates. To 100µl to 200µl of varying concentrations of the extracts, 100µl of nutrient broth was added and then a loopful of the test organism previously diluted, the micro well plate was incubated for 24hrs and each dilution was plated and observed for the bacterial growth inhibition. The MIC values were interpreted as the highest dilution of the sample, which showed no growth.

Table 1: Biochemical tests to identify bacterial isolates.

TEST	SAMPLE - 1	SAMPLE - 2	SAMPLE - 3
Gram staining	Negative rods	Negative rods	Positive rods
Motility	Motile rods	Motile rods	Motile rods
Indole	- ve	-ve	-ve
MR	- ve	- ve	-ve
VP	+ve	-ve	-ve
Urease	- ve	-ve	+ ve
Citrate	+ve	+ve	+ ve

RESULTS AND DISCUSSION

Selection of PHB producing bacterial isolates from soil samples
Among the several bacterial isolates from soil, three showed positive result for PHB production by sudan black dye as shown in fig.1. Hence they were chosen for the study.

Fig. 1: Bacterial isolates from different soil samples tested positive for PHB production.

Identification of the isolates based on biochemical tests: Based on the biochemical tests as shown in table 1, the three isolates were identified as *Enterobacter sp.*, *Pseudomonas sp.* and *Bacillus sp*.

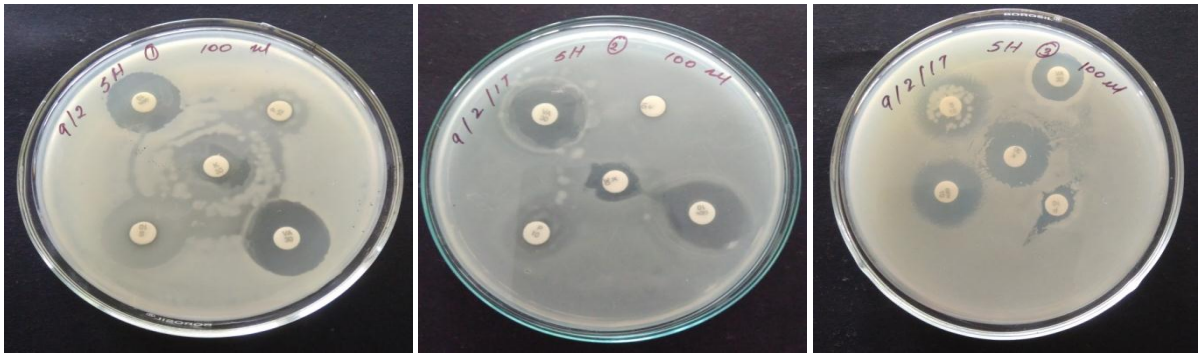
Antibacterial activity: Their sensitivity to various antibiotics was evaluated by disc diffusion assay. Results from tables 2&3 revealed that isolates 1&2 were sensitive to all the 5antibiotics used in the study, whereas isolate 3 was found to be multidrug resistant showing no zones of inhibition for streptomycin, penicillin and kanamycin (fig.2).

Table 2: Effect of a few antibiotics at 100 µl on the chosen isolates.

Antibiotic	Sample -1	Sample - 2	Sample - 3
Gentamycin	20 mm	15 mm	22 mm
Kanamycin	21 mm	11 mm	7 mm
Vancomycin	21 mm	20 mm	15 mm
Streptomycin	20 mm	9mm	6 mm
Penicillin	15 mm	7mm	-

Table 3: Effect of a few antibiotics at 200 µl on the chosen isolates.

Antibiotic	Sample -1	Sample - 2	Sample - 3
Gentamycin	19 mm	15 mm	16 mm
Kanamycin	20 mm	18 mm	-
Vancomycin	22 mm	16 mm	14 mm
Streptomycin	15 mm	10mm	-
Penicillin	15 mm	9 mm	-



Sample 1 **Sample 2** **Sample 3**

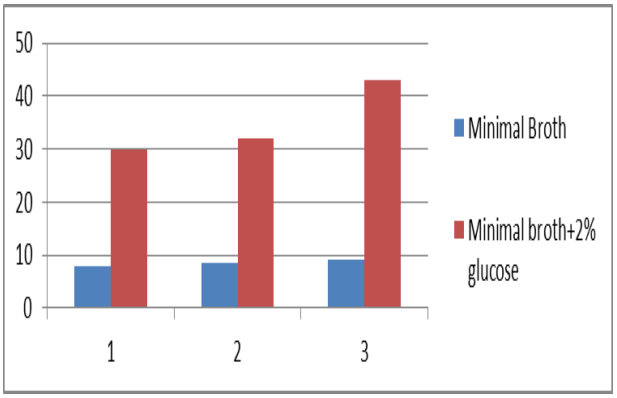
Fig. 2: Plates showing Zones of inhibition by the antibiotics against the bacterial isolates.

3.4. PHB Production

Polyhydroxy butyrate production of the 3 isolates were determined in both minimal broth and also in minimal broth supplemented with 2% glucose. The PHB production after 24hr incubation was estimated by determining the OD at 230nm as shown in table 4. Among the 3 bacteria chosen for the study, isolate 3 was the predominant producer of PHB. About 43µg of PHB was produced by the 3rd isolate in glucose supplemented medium in contrast to only 9µg in minimal broth. This increase in PHB production is depicted in graph.

Table 4: Estimation of polyhydroxybutyrate production.

Sample	PHB production in µg Minimal broth	Minimal broth+2% glucose
1	8	30
2	8.5	32
3	9	43



Graph 1: Depicting the PHB production by the three isolates in minimal medium and minimal medium with 2% glucose.

It is also interesting to note that the 3rd isolate which is the predominant PHB producer was also resistant to 3 antibiotics. The correlation between the resistance and PHB production is yet to be analyzed. This has not been reported earlier. The significance of this relationship is under study. The reason for increased production of PHB in 2% glucose supplemented media justifies the mechanism of PHB production, where sugars are converted to acetyl co-A and to 3hydroxy butyryl leading to PHB production by PHB synthase. PHB has a wide range of potential applications because of its desired features such as biocompatibility, biodegradability and

negligible cytotoxicity to the cells. Hence, the potential application of PHB as replacement for petrochemical based polymers is gaining popularity in various fields involving packaging, medical and coating materials. These desirable properties in compounding and blending have broadened their performances as potential end-use applications. Development of PHB as potential substitute material to some conventional plastics has drawn much attention due to the biodegradable and biocompatible properties of PHB. Because of its good biodegradability and biocompatibility, PHB has potential use in advanced drug delivery systems. However, PHA production strains are still needed. The lipophilic staining with Sudan Black B (SB staining) reportedly has high sensitivity in PHB screening (Nighat Naheed, 2011). After SB staining, bacteria containing PHAs exhibit dark granules. Therefore, Sudan Black B staining is a simple method of screening potential strains for PHBs. The Nitrogen source depletion leads to the cessation of protein synthesis which in turn leads to the inhibition of TCA cycle enzymes (citrate synthase and isocitrate dehydrogenase) and consequently slows down the TCA cycle. As a result, the acetyl Co-A routes to PHB synthesis (*de* Lima TS, 1999). The main limitation in the commercial production of PHB as biodegradable plastic is its high production costs compared to the production of petrochemical based synthetic plastics.

CONCLUSION

Among the three isolates identified for PHB production, the third isolate identified as Bacillus sp. exhibited resistance against three of the five antibiotics tested. It is also interesting to note that the same isolate could produce the maximum PHB when compared to the other two isolates. The correlation between the resistance and PHB production is yet to be analyzed. This has not been reported earlier. The significance of this relationship is under study.

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