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SCREENING AND IDENTIFICATION OF EFFICIENT POLYHYDROXYBUTYRATE PRODUCING BACTERIA FROM PLASTIC WASTES DUMPED SOIL SAMPLES.

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ABSTRACT

The soil samples were collected from the polythene and plastic waste dumped site area of Sankarapuram. The collected samples were subjected to serial dilution and plating technique. The isolated microbial strains were identified based on their cultural morphological and biochemical study. To identify the PHB producers from non-producers Carbol fuchsin staining was carried, for this staining *Bacillus* species found to have dark colored granules within their cells. For further confirmation PHB producers were stained with Sudan black, the results observed as dark black to purple granules against pink back ground when counter stained with Safranin. Polythene bag degradation (broth and tray method) by *Bacillus species* were observed for one month. After the incubation color changes softness of plastics were observed due to the degrading action of microbes. The green gram seeds were inoculated in 3 pots each pot treated with *Bacillus* strain I, II, III, the growth rate were analyzed. Compared with other two strain the *Bacillus megaterium* gave the maximum growth. The FT-IR analysis identified the functional groups.

KEYWORDS: Plastic degrading microbes, PHB producers and FT-IR analysis.

INTRODUCTION

The ecological problems related to the environmental pollution by synthetic polymers like plastics are one of the major concerns of the present day especially because they are difficult to degrade easily and the entire process is time consuming. The environmental concerns include air, water and soil pollution are also in effect under regulated condition biodegradable

plastics could potentially degrade to the point where microorganisms can metabolize them tolerant, non halophilic microorganisms from food samples and evaluation of their characteristics under stress condition. Microorganisms produce some useful compounds that are important part of the biological balance in the life in our planet.[1]

Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer made of long chain monomers of ethylene. The worldwide utility of polyethylene is expanding at a rate of 12% per annum and approximately 140 million tons of synthetic polymer are produced worldwide each year with such huge amount of polyethylene getting accumulated in the environment, their disposal evokes a big ecological issue, It take thousand year for their efficient degradation. Biosurfactant, an extracellular surfactant secreted by microorganisms, enhances the biodegradation process.[2,3]

Biodegradable polymers are designed to degrade upon by the action of living organisms. Biodegradable polymers generally decompose in various medium in our environment. The depolymerisation results due to various physical forces such as temperature, moisture, pressure etc., deal with causing mechanical damage to the polymer. The microbial biodegradation is widely accepted and is still underway for its enhanced efficiency. Recently several microorganisms have been reported to produce polyester degrading enzymes. The microbial species are associated with the degrading materials were identified as bacteria (*pseudomonas*, *Streptococcus*, *staphylococcus*, *micrococcus* and *Moraxella*), fungi (*Aspergillus niger*, *Aspergillus glaucus*) and Actinomycetes species. Microbial degradation of plastic is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized. Aerobic metabolism results in carbon dioxide, water and whereas anaerobic metabolism results in carbon dioxide, water and methane as the end products, respectively. The degradation leads to breaking down of polymers to monomers creating an ease of accumulation by the microbial cells for further degradation.

Microbial degradation of plastic is caused by enzymatic activates leading to chain cleavage of the polymer into oligomers and monomers after which they further metabolized by the microbial cells. Aerobic and anaerobic results in carbon dioxide and water, and methane as the end products, respectively.[4]

MATERIALS AND METHODS

Collection of Soil Sample

Soil sample were collected from polythene and plastic dumped sites of Sankarapuram, Villupuram District, Tamil Nadu.

Serial dilution technique (booth and morelb, (2006)

Serial dilution was performed by using the collected soil samples to isolate the sample bacteria.1 gram of soil sample were diluted in the test tube containing 9 ml of sterile distilled water and mixed thoroughly to make a 1:10 dilution (10-1). The 1 ml of diluted sample was transferred to the next test tube and serially diluted into the serious of test tube, having 9 ml of sterile distilled Water with sterile pipettes up to 10^{-6} dilution.[5]

Isolation of Bacteria from soil sample

Identification of the microorganisms

The collected sample microorganisms were grown in nutrient medium. The medium is defined as the substrates in which microorganisms could grow and multiply. For the present study, synthetic medium was used for culturing, conducting biochemical test and antibiotic sensitivity purpose. Synthetic media was one in which all the constituents are chemically defined. They are used to study the specific nutritional requirements of the microbes and following tests were conducted to identify the organisms.

Culture examination

Once the growth was found primary plate, then the identification was carried out by the following systematic method, for examining the type of colony, color change in the medium, morphology of the cells under stained and unstained conditions and biochemical tests were done by using Berges's manual.

Carbol fuschsin staining

Carbol fuschsin staining was performed to determine the intracellular production of PHB by the isolate, a thin smear of all the isolated were stained with Carbol fuschsin stain for 45 seconds. The isolates capable of producing PHB showed dark color granules of PHB interacellularly.[6]

Sudan black Staining

PHB producing bacteria was further confirmed using Sudan black B staining method with some minor modifications; Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol.[7]

Procedure

The smear of culture was prepared on glass slides and heat fixed. The samples were stained for 10 min with Sudan black solution, rinsed with water and counter stained with 0.5% Safranin for 5 min and observed at 1000x Phase contrast microscope.

Chi-square Test

The chi-square test was applied. The purpose of Chi square test was decided to whether the set of observed date (anti-bigram of microorganisms) agrees with the standard antimicrobial disc susceptibility test.[8]

Collection of plastic

Polythene bag were collected from local market in Sankarapuram, Villupuram District, Tamil Nadu, India.

Microbial degradation of plastic in laboratory condition

Nutrient medium preparation

The nutrient broth medium was prepared in 3 conical flask each contains 50 ml, Then the medium was sterilized with autoclaving 121°C for 15 minutes.

Inoculation of organisms

Isolated plastic degrading microorganisms were inoculated into the medium containing plastics. The results were observed for 1 month.

PLASTIC DEGRADING USING TRAY METHOD

Collection of polythene bags

Different types of polythene bags were collected from local area in Sankarapuram Villupuram District, Tamil Nadu, India.

Collection of soil

The soil samples were collected from Vellimalai, Sankarapuram Villupuram District, and Tamil Nadu.

Tray method

The collected soil sample was sterilized and the soils were transferred to trays. The soil was mixed with isolated plastic degrading microbes and 50 ml of sodium acetate solution were added with the soil. The collected plastic bags were making into pieces and inoculated into the trays.

The trays were covered with the polythene sheets and maintain in the room temperature. After 1 month the degradation of inoculated plastic bags were observed.

RESULT AND DISCUSSION

The present study deals with the isolation of plastic degrading microbes by using enrichment medium. All the isolates were subjected to various tests to nitrogen fixing confirmation to identify.

In this study, plastic dumbed site soil sample from Sankarapuram hills area were selected for the isolation of bacteria species using serial dilution and plating methods. Serially diluted sample were poured into the nutrient agar plates (10^{-4} to 10^{-6}) totally 9 isolates were selected. Due to the efficiency of the strain we were choosing 3 strain for future research.

Identification of plastic degrading microbes

In the present study Gram positive, spherical, motile approximately 1m in diameter arranged characteristically in cluster showing negative results for Indole, MR, Citrate and positive results for catalase and oxidase test, alkaline and acid butt formation on TSI agar were identified as *Bacillus subtillis*.

In the present study found that Gram positive, spherical, motile approximately 1m in diameter arranged characteristically in cluster showing negative results for Inodle, VP, Citrate, alkaline and acid butt formation of TSI agar were identified as *Bacillus megaterium* (Table 1).

To distinguish PHB producers form non- producers carbol fuschsin staining was carried out. Upon staining with carbol fuchsin *Bacillus species* were found to have dark colored granules of PHB within their cells.

In earlier study also found that plastic degrading microbes isolated from forest soil to determine the plastic degrading of Micrococcus luteus and Masoniella sp. The previous

study reported that the several bacteria isolated from soil sample were studied for PHB production as soil is rich in micro flora. Various colony morphologies including rhizoidal and branched colonies typically for *Bacillus* species were obtained. The bacteria were initially screened for the production in basel mineral salt broth and ability to synthesize PHB granules was confirmed using nile blue and nile red staining of PHB ganules in the intracellular environment of the isolated bacteria. Based on the intensity of the fluorescence observed in the staining method the potential PHB producers were identified.[9,10,11]

In the present study identify the PHB producing *Bacillus* strains of earlier study noticed that all the bacterial isolated were able to produce substained amounts of PHB during growth using the simplified media mentioned above containing a single carbon and nitrogen source. The PHB accumulation was noticed as early as 16 hours of incubation in the bacterial cells. The synthesis of PHB was noticed from the log phase of growth and it continued until late exponential phase the carbon source was utilized for both growth and PHB production.

Infrared spectrum analysis

In the present study showed that the analysis of infrared spectrum of *Bacillus sp* were identified. The FT-IR analysis of the purified metabolites of *Bacillus subtillis*, showed the presence of alkanes, aromatic compound, alcohol, and aldehyde and ketone. *Bacillus subtillis* 2 showed the presence of alkanes, Aldehyde and ketone. *Bacillus megaterium* showed the presence of Alkanes, aldehyde, ketone, alcohol, halogen and aromatic compounds. Hence the metabolites have capacity to inhibit the pathogenic bacteria. (Table 2,3,4,5 and 6).

The earlier study reported that the FTIR spectroscopy is used as analytical technique in many biodegradation studies. It is a useful tool to determine the formation of new or disappearance of functional group.[12]

Polythene bag degradation (broth and tray method) by *Bacillus* species were observed for one month. After the incubation color changes softness of plastic were observed due to the degrading microbes. (Table 7 and plate 1, 2).

The green gram seeds were inoculated in 3 pots each pot treated with *Bacillus* strain 1, 2, 3, the growth rate were analyzed for 1 month . The results were compared with other two strain the *Bacillus megaterium* gave the maximum growth. (plate 3).

Plate 1. Analysis of PHB Producing bacteria using degradation of Plastics (Tray method).

Collection of plastic bags

Inoculation and Incubation of plastic bag into the *Bacillus* species with soil sample and Charcoal (I month).

Plate 2. Analysis of PHB Producsing bacteria using degradation of Plastics (Broth method).

Inoculation of plastic bag into the nutrient broth with *Bacillus* species.

Plate 3. Analyzing of plant growth (15th & 30 th day).

Analysis of plant growth in 15 days.

Analysis of plant growth in30 days.

Table 1. Morphological and Biochemical Characteristic of isolated organisms.

S.NO	Characterization	*Bacillus subtillis*	*Bacillus megaterium*
1	Colony texture	Smooth	Large
2	Gram's reaction	+	_
3	Shape	Small Rod	Cocci-bacilli
4	Indole	+	_
5	Methyl red	+	+
6	Voges proskauers	+	_
7	Citrate Utilization	_	_
8	TSI	Acid slant +	Acid slant _
9	Nitrate	+	_
10	Urease	+	+
11	Casein	+	+
12	Catalase	+	+

Table 2. Infra-red spectrum Analysis of *Bacillus subtillis* first day.

S.No	PEAK VALUE	STRETCHING	INTERPRETATION
1	3913.44	C-H	Alkanes
2	3435.32	C-H	Alkanes
3	2078.30	C-H	Alkanes
4	1637.45	C=C	Alkanes
5	1444.20	C-H def	Aromatic compounds
6	1358.65	C-H def	Aromatic compounds
7	1637.45	C=C	Alkanes
8	1117.59	C-O	Alcohol

9	1020.98	C-O	Alcohol
10	682.40	C-H def	Alkanes

Table 3. Infra-red spectrum Analyse of *Bacillus subtillis* (2) first day.

S.No	PEAK VALUE	STREATCHING	INTERPERTATION
1	3764.30	C-H	Alkanes
2	3434.08	C-H	Alkanes
3	2381.35	C-H	Alkanes
4	2078.23	C-H	Alkanes
5	1811.54	C=O	Aldehydes and ketone
6	1637.10	C=O	Aldehydes and ketone
7	1407.52	C-O	Alcohols
8	1362.56	C-O	Alcohols
9	1227.86	C-O	Alcohols
10	1100.42	C-O	Alcohols
11	680.56	C-Cl	Halogen

Table 4. Infra-red spectrum analyse of *Bacillus megaterium* first day.

S.No	PEAK VALUE	STREATCHING	INTERPERTATION
1	3914.94	C-H	Alkanes
2	3765.03	C-H	Alkanes
3	3437.96	C-H	Alkanes
4	2391.48	C-H	Alkanes
5	2080.30	C-H	Alkanes
6	1811.69	C=O	Aldehydes and ketone
7	1637.87	C=O	Aldehydes and ketone
8	1439.59	C-O	Alcohols
9	1410.52	C-O	Alcohols
10	1356.98	C-O	Alcohols
11	1112.10	C-O	Alcohols
12	677.08	C-Cl	Halogen

Table 5. Infra-red spectrum analyses *Bacillus subtilis* (2) third day.

S.NO	PEAK VALUE	STREATCHING	INTERPERTATION
1	3908.73	C-H	Alkanes
2	3755.87	C-H	Alkanes
3	3433.47	C-H	Alkanes
4	2372.32	C-H	Alkanes
5	2339.38	C-H	Alkanes
6	2080.24	C-H	Alkanes
7	1854.11	C=0	Alcohol
8	1637.89	C-C	Alkanes
9	1441.84	C-Hdef	Aromatic compounds and ketone
10	1406.51	C-Hdef	Aromatic compounds and ketone
11	1358.01	C-Hdef	Aromatic compounds and ketone
12	1089.80	C-O	Alcohols
13	680.61	C-Hdef	Aromatic compounds and ketone

Table 6. Infra- red spectrum analyse *Bacillus megaterium* third day.

S.No	PEAK VALUE	STREATCHING	INTERPERTATION
1	3918.23	C-H	Alkanes
2	3441.08	C-H	Alkanes
3	2392.01	C-H	Alkanes
4	2081.76	C-H	Alkanes
5	1638.41	C=C	Alkanes
6	1414.63	C-Hdef	Aromatic compounds and ketone
7	1436.26	C-Hdef	Aromatic compounds and ketone
8	1358.95	C-Hdef	Aromatic compound and ketone
9	1109.81	C-O	Alcohols
10	676.60	C-Hdef	Aromatic compounds and ketone

Table 7. To analyses the weight loss of plastic degrading microbe for 1 month (broth and tray method).

Strain no.	Initial wt. (mg)	Final wt. (mg)	Difference	Weight loss/month (in%)
Bacillus subtillis	1	1	NIL	No changes
Bacillus subtilis(2)	1	1	NIL	No changes
Bacillus megaterium	1	1	NIL	No changes

CONCLUSION

The present study we concluded that the *Bacillus* strain I, II &III were used to analyzes the growth of the plant , compared with the other two strain of the *Bacillus* species the *Bacillus megaterim* gave the maximum growth of the plant. The plastic degrading microbes can control the bacterial phytopathogens without any effects in plants and fix the nitrogen to the plant growth.

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