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ISOLATION AND OPTIMIZATION OF CULTURE CONDITIONS FOR PHB PRODUCTION BY *BACILLUS MEGATERIUM*

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ABSTRACT

Poly-β-hydroxybutyrate is an irresistible replacement to petroleum based synthetic plastic, because has mechanical properties similar to polypropylene and completely biodegradable too. In current piece of work, an attempt was made to isolate potent PHB producing bacteria from the different root nodule, soil and water samples. Sudan Black B was used for primary screening of bacterial isolates for PHB production. Presence of functional groups in extracted PHB was confirmed with the help of FITR analysis. Culture media conditions having incubation time period 72 h, temperature 30˚C, pH 7, glycerol as a carbon source, ammonium sulphate as nitrogen source and C:N ratio as 16:1 were found to be supportive for maximum PHB production by isolate M.Com2(1). Isolate M.Com2(1) (*Bacillus megaterium*) exhibited significant PHB yield, so that can be exploited for use in industries.

KEYWORDS: **Polypropylene, Biopolymer, Sudan Black and FTIR analysis.**

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INTRODUCTION

Synthetic plastic is an exquisite gift of present day science and technology for humanity. This wonderful product has some exclusive qualities of being light as well as strong and reasonably priced. Plastic play important role in our day to day life like it use to prepare simple plastic carry bags to complex surgical medical devices[1]. Petroleum based synthetic plastic derive from insubstantial non renewable natural resources and led serious environmental problems because of their non biodegradable nature. As considering the seriousness of problems caused by petroleum based synthetic plastic there is a strong need to develop an environment friendly and cost effective biodegradable natural material is required[2]. Among biopolyners Poly-β-hydroxybutyrate is best alternative of synthetic plastic because its thermoplastic characteristics are similar to the synthetic polymer to a large extend. PHA is biodegradable, compostable and biocompatible[3]. Poly- β-hydroxybutyric acid (PHB) is a most valuable member of a poly- β-hydroxyalkanote (PHA). PHB is produced by several microorganisms such as *Bacillus megaterium*, *Ralstonia eutrophus, Cupriavidus necator, Rhizobium* spp., *Azotobacter* spp., *Pseudomonas* spp., etc[4]. PHB is synthesized by cells under limited growth conditions like carbon in excess and other growth factors such as nitrogen, phosphorus or sulfur are in limiting concentration. Accumulation of intracellular storage of PHB has been considered as part of survival mechanism used by microbes in adverse conditions[5]. The major limitation in use of PHB is high production cost that creates difficult situation for the use of PHB at commercial level. In the last few years considering the environmental deterioration various attempts have been made to use the PHB for replacement of synthetic plastic at industrial level. Therefore alternative strategies such as isolation of potent PHB bacteria and optimization culture condition parameters for maximum PHB production are required for reducing the cost of PHB production. Hence keeping above facts in consideration, this project was designed to isolate PHB producing bacteria from various leguminous root nodules, soil and water samples and to optimize culture conditions such as incubation period, temperature, pH, carbon source, nitrogen source and ratio of carbon and nitrogen source for increase in PHB production and characterization of extracted PHB.

MATERIALS AND METHODS

Sample Collection

Samples were collected from different sites in Rajasthan. For isolation of PHB producing bacteria Samples were collected from root nodules of leguminous plants, soil and water sample from various sites like soil samples were taken from rhizosphere, composting site, municipal waste site, mud soil, ground of Mody University, Lakshmangarh (Rajasthan) and water samples were taken from house hold water, sewage water, waste water, salt water (Table I). Root nodules and soil samples were stored in plastic bags and water samples were in glass bottles at 4˚C temperature. A standard PHB positive bacterial strain, *Cupriavidus necator* MTCC 1472 was selected as a positive control which was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Isolation of Microbial strains

Root nodules were sterilized with 0.1% mercuric chloride, grind and a full loop of smash nodules was streaked on YEMA medium plates. Selected colonies were sub cultured to obtain pure culture. The soil and water samples were subjected to serial dilution plating. For that 10 fold dilutions were made ranging from 10^{-1} to 10^{-7}. Dilutions were plated on nutrient agar plates. After 48 hrs of incubation at 30˚C, well formed colonies were re-streaked to obtain pure culture. Pure culture was maintained in agar slants.

Screening of isolates for PHB production

Selected pure bacterial isolates were screened for PHB production using carbol fucshin and lipophilic Sudan Black B Stain.

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Carbol fuchsin staining

Carbol fuchsin staining was performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolated were stained with carbol fuchsin stain for 45 s. The isolates capable of producing PHB showed dark colored granules of PHB intracellularly[6].

Sudan black B 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]. The smear of cultures 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 magnification.

PHB extraction and quantification

Polyhydroxybutyrate polymer was extracted using the dispersion method with few modifications[8, 9]. Bacterial cells were collected by centrifugation at 10,000 rpm for 10min at room temperature. Pellet was washed with phosphate buffer saline (pH 7.4). Cell pellet were air dried and their weights were taken. Chloroform and sodium hypochlorite were added to the cell pellet in a ratio of 12.5µl Chloroform and 12.5µl sodium hypochlorite per mg of pellet weight. The mixture was kept overnight at room temperature. That was then centrifuged at 8000rpm for 10 min at room temperature resulting in the formation of different phases. The bottom phase of chloroform contains PHB. This phase was transferred to another fresh tube and its volume measured. 3 x volume of methanol was added to chloroform solution. The mixture was centrifuged at 10,000 rpm for 15 min resulting in the formation of a precipitate of PHB. The amount of PHB present was quantified by determining the weight of precipitate obtained. The supernatant was discarded and the pellet was dissolved boiling concentrated sulfuric acid results in brown color solution.

FTIR analysis of PHB extracts

FTIR is one of the important techniques to obtain the information regarding structure of the compound. The extracted polymer samples were prepared in KBr pellet and FTIR absorption spectrum was recorded in a range of 4000 cm^{-1} to 400 cm^{-1}. The FTIR spectra of the bacterial isolates were compared with that of the standard PHB purchased from Sigma Aldrich[10].

Morphological, biochemical and Molecular characterization of PHB positive isolates

The PHB producing isolates were grown on nutrient agar plates and their colony morphology was recorded. The morphological characteristics of the selected bacterial isolates were recorded under four major headings likewise size, colour, texture and shape. All these isolates were also studied under the microscope with respect to their cellular morphology and Gram staining properties[11]. Biochemical characteristics of the isolates were studied following the standard microbiological methods[12]. Molecular identification was checked by 16S rDNA sequencing[13]. Genomic DNA was isolated from PHB positive bacterial isolates and gene amplification was done by PCR using universal primers.Identification of isolates was carried out on the basis of the results of morphological, biochemical and molecular characters studied.

Culture condition optimization for increase in PHB production

Effect of different incubation periods on PHB production

The isolates were incubated for different time periods ranging from 24 to 96 h. At each point of time (24, 48, 72 and 96), cultures were tested for bacterial growth and PHB production[14].

Effect of Different temperatures on PHB production

To standardize the optimum temperature for PHB production, bacterial isolates were inoculated in M9 broth at temperatures 26˚C, 30˚C, 37˚C and 42˚C for 72 hours. PHB yield was quantified, based on that optimum

temperature for the maximum PHB production was determined[15].

Effect of different pH on PHB production

The effect of different pH on PHB yield was checked by inoculating cultures in M9 medium having different pH viz., 5, 6, 7, 8 and 9. Cultures were incubated in shaking condition at 30˚C at 100 rpm for 72 h. After incubation PHB production was checked with chloroform dispersion method and the optimum pH for maximum PHB yield was determined[15].

Effect of different carbon sources on PHB production

The effect of various carbon sources on PHB production was determined by inoculating the cultures in M9 medium supplemented with glucose, sucrose, starch, mannose and glycerol at 1% concentration. Cultures were incubated at 30˚C on a shaker with 100 rpm for 72 h. Based on the maximum PHB yield the best carbon source was determined[16].

Effect of different nitrogen source on PHB production

The PHB positive isolates were inoculated in 30ml of M9 broth containing the best carbon source and different nitrogen sources (tryptone, peptone, ammonium sulphate, cystein and soyabean meal) at 0.5% concentration. PHB yield was determined for all the isolates after 72 h of incubation at 30˚C, and the best nitrogen source was selected on the basis of their yield[16].

Effect of different Carbon to Nitrogen Ratio on PHB production (C/N Ratio)

In addition to the determination of the best C and N sources, the effect of different C: N ratios on PHB production were also determined. For this, cultures were inoculated in M9 supplemented with different ratios of concentrations of the best C and N source (C/N ratio as 1:1, 2:1, 4:1, 8:1, 16:1 and 20:1). Cultures were incubated at 30°C on a rotary shaker 100 rpm for 72 h. After incubation, PHB yield was quantified based on the yields the most favorable C/N ratio was determined.

RESULTS AND DISCUSSION

Sample collection and Isolation of PHB producing bacteria

A total of 96 well formed bacterial colonies (10 from root nodules, 7 from rhizospheric soil sample, 9 from composting site soil, 4 from house hold water, 7 from waste water sample, 12 from sewage sample, 4 from salt water sample, 1 from municipal waste soil, 9 from Mody University ground soil and 10 from mud soil sample) were picked, re-streaked and pure cultures were maintained for further analysis.

Screening of PHB producing strains

To differentiate PHB producing bacteria from non PHB producing bacteria, carbol fuchsin staining was carried out. Twenty one isolates were found have dark colored granules of PHB within their cells after carbol fuchsin staining[17]. For further confirmation, these isolates were stained with Sudan Black B dye[18]. Purple to black granules were observed intracellularly with a pink background (Figure I). This represents the PHB producing isolates.

PHB extraction and quantification

All the selected 21 isolates and the standard strain were subjected to quantitative estimation of PHB by dispersion method[8]. The yield of PHB production was found to vary between 1.5% (w/w) to 16.09% (w/w), by isolate MK9 and M.Com2(1) respectively (Table II). Highest PHB producing bacterial isolate was obtained from composting soil sample and the isolate with lowest yield belong to mud soil sample. Although, the PHB yield of standard strain was found to be higher than the isolates.

FTIR analysis of extracted PHB

In this present study PHB extracted from various isolates were observed by Fourier Transform Infra Red (FTIR) spectrum[19]. The standard PHB showed strong bands at 3430cm^{-1}, 2976cm^{-1} and 1724 cm^{-1} which represents the presence of terminal OH group, methylene C-H vibrations and carbonyl group respectively (Figure II). The selected PHB sample was revealed almost similar peaks with standard,

whereas the remaining peaks are closely lying between 3430cm^{-1} to 400cm^{-1} (Figure III) [20, 21].

Morphological, biochemical and Molecular characterization of PHB positive isolates

Out of 21, highest PHB producing bacterial isolate was selected for further screening. So morphological, biochemical and molecular characterization was checked for selected M.Com2(1) isolate. Colony morphology was recorded in terms of size, shape, texture, colour and staining characteristics. Large sized, circular shaped, off white coloured and raised colonies was observed. Purple colored rod shaped cells were observed. They were thus found to be Gram positive. The selected PHB producing isolate was subjected to standard biochemical tests and characterization was carried out as per details given in Bergey's Manual of Systematic Bacteriology[22]. On the basis of that the isolate has been found to belong to genera *Bacillus* (Table III). For molecular identification genomic DNA of bacterial isolate M.Com2(1) was isolated because it is a highest PHB producer among all screened isolates. The isolate was having DNA of more than 1Kb size. Purified genomic DNA of these isolates was amplified using the universal primers for 16S rDNA. Sequencing was done through Shrimpex Biotech Services Pvt. Ltd., Chennai. The sequences were identified by using NCBI BLAST.

Culture condition optimization for increase in PHB production

Effect of different incubation periods on PHB production

PHB was being produced and with increase in incubation its consumption will increase[23]. The effect of incubation time on PHB produced by isolate was shown in figure IV. 72h was found to be an optimum incubation period for M.com2(1). The isolate was found to produced a PHB yield of 15.88% (w/w).

Effect of different temperatures on PHB production

Effect of different incubation temperatures were checked on PHB production. 30˚C temperature was found to be optimum for maximum PHB production by all the selected isolate. The maximum PHB yield was found to be 16.09 % (w/w). Below and above this temperature decrease in PHB production was shown (Figure V). Similar results have been shown in previous studies[24]. There it has been shown that 33˚C temperature was optimum for PHB production.

Effect of different pH on PHB production

A range of 5 to 9 media pH was checked to find out optimum pH for PHB production. pH 7 was found to be optimum for maximum PHB production by M.com2(1) (Figure VI). At pH 5 and pH 9, the selected isolate was found to produce very low yield of PHB, which revealed that acidic and basic media is not suitable for high yield of PHB. At pH 7, the highest PHB yield of 16.21% (w/w) was produced by M.Com 2(1) which was significantly higher than all the other isolates. The effect of culture media pH on PHB yield was also studied and the maximum production obtained at pH 7[24].

Effect of different carbon sources on PHB production

The effect of various carbon sources (glucose, sucrose, starch, mannose and glycerol) on PHB yield was shown in figure VII. Among the carbon sources tested glycerol was found to be the best carbon source for M.com2(1)[25].On the basis of the previously reported data and according to the result obtain in present study, it can be concluded that simple sugars like glycerol and glucose are easily utilized by bacteria and because of that enhance the growth and PHB production simultaneously. Although, complex molecule like starch not readily utilized by significant PHB production. Similar conclusions have been made in earlier studies[26]. The maximum PHB production 18.81% (w/w) was again shown by M.Com2(1).

Effect of different nitrogen on PHB production

The effect of different nitrogen sources (tryptone, peptone, ammonium sulphate, cystein and soyabean meal) on PHB yield was represented in figure VIII. M.Com 2(1) produced 17.92% (w/w) PHB. Among the

various nitrogen sources used ammonium sulphate was found to be best nitrogen source[26]. These results are supported by the results maintained in earlier studies[27], there *Ralstonia eutropha* was produced maximum PHB when grown in production media supplemented with ammonium sulphate. Ammonium sulphate is simple nitrogen source and easily available rather than other complex nitrogen sources.

Effect of different Carbon to Nitrogen Ratio on PHB production (C/N Ratio):

Figure IX shows the PHB yield shown by M.Com 2(1) isolate in the presence of different carbon to nitrogen ratio. Among the various carbon to nitrogen ratio tested, 16:1[28] was found to be optimum carbon and nitrogen ratio supporting the maximum PHB production. There was an increase in C:N ratio upto 16:1and after that decreasing. Substrate inhibition might be a reason behind it. Similar observations have been made in previous studies[29].The selected isolate was found to be produced PHB yield of 24.54 %(w/w).

Figure I
PHB granules stained by Sudan Black B staining

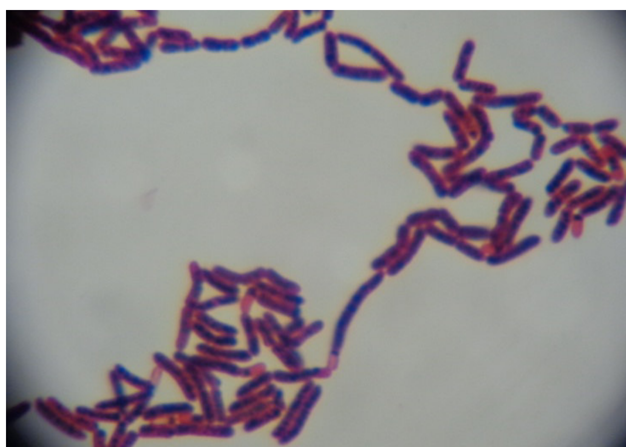

Figure II
FTIR spectra of standard PHB

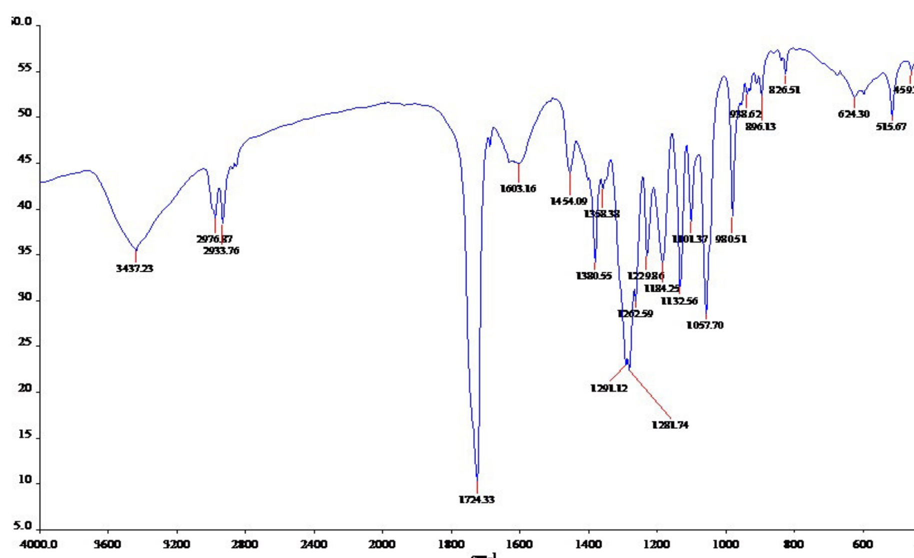

Figure III
FTIR spectra of PHB extracted from M.Com2(1) isolate

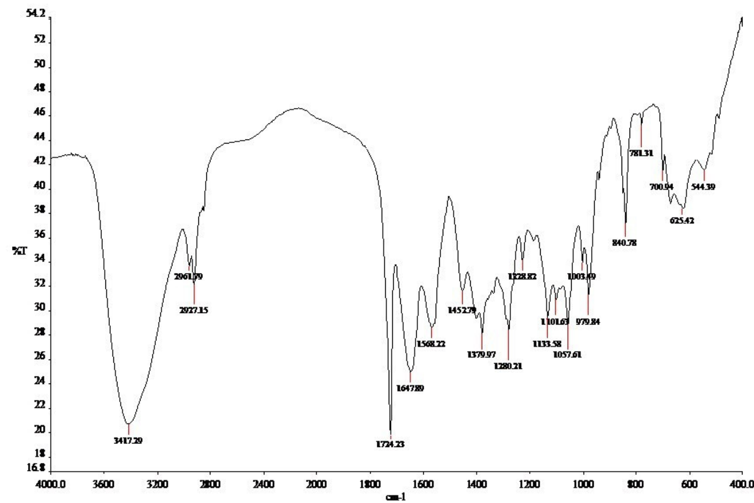

Figure IV
Effect of incubation time on PHB production by M.Com2(1) isolate

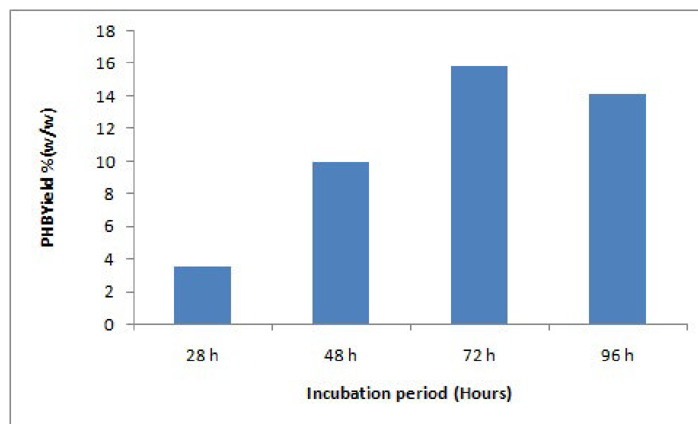

Figure V
Effect of temperature on PHB production by M.Com2(1) isolate

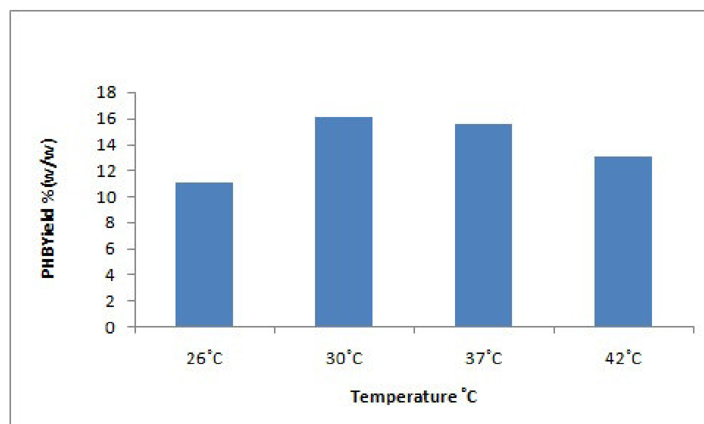

Figure VI
Effect of pH on PHB production by M.Com2(1) isolate

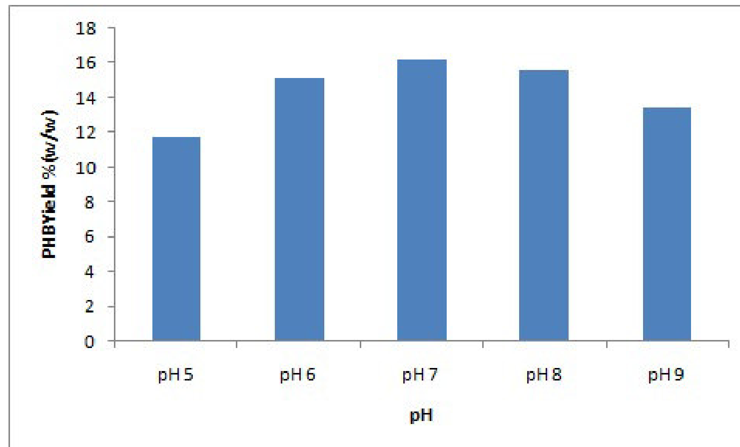

Figure VII
Effect of different carbon source on PHB production by M.Com2(1) isolate

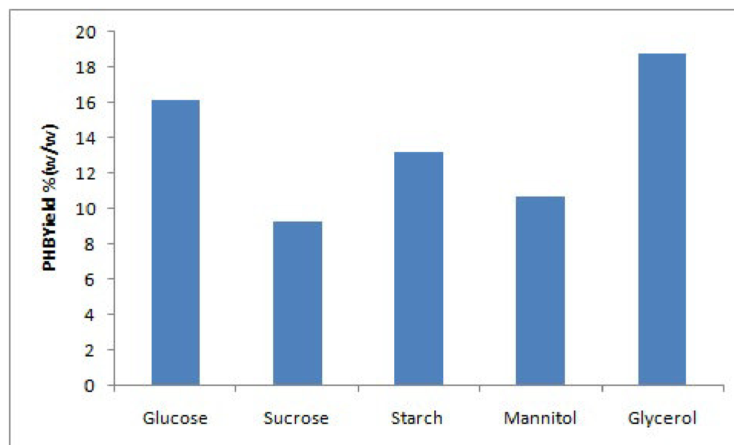

Figure VIII
Effect of different nitrogen source on PHB production by M.Com2(1) isolate

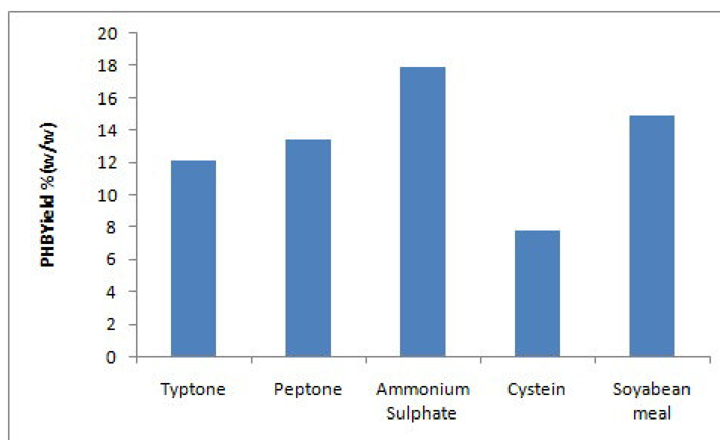

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Figure IX
Effect of different C:N ratio of media on PHB production by M.Com2(1) isolate

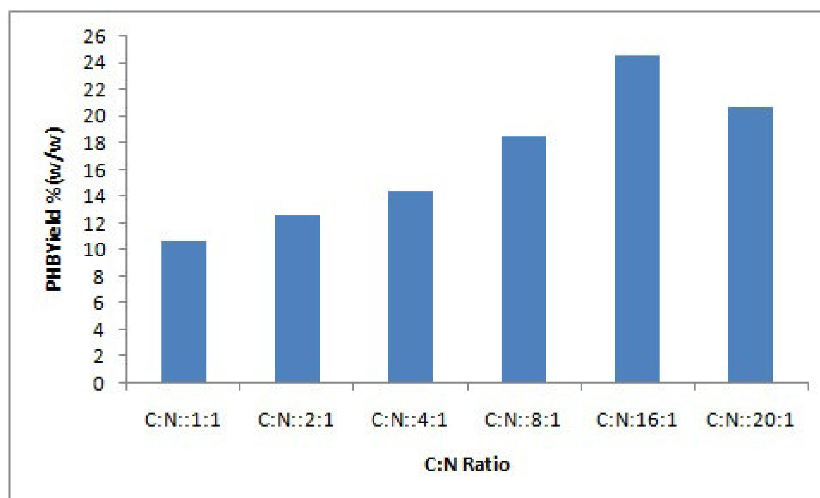

Table I
Isolation and Screening of PHB producing isolates

S.No.	Sample description	No. of sample tested	No. of representative isolates	No. of PHB granules containing strains
1.	Root nodules	7	10	3
2.	Grass Rhizospheric soil	2	7	2
3.	Composting site	2	10	2
4.	House hold water	2	4	0
5.	Waste water	2	7	1
6.	Sewage	1	12	4
7.	Salt water	2	12	4
8.	Municipal waste soil	2	16	1
9.	Soil from Mody University	1	9	2
10.	Mud soil	2	10	2

Table II
PHB production by selected isolates

S. No.	PHB positive isolates	PHB yield% (w/w)
1.	M7(0)	11
2.	M7(2)	13
3.	M7(3)	7
4.	MK9	1.5
5.	MK9(1)	5.3
6.	M.Com2(1)	16
7.	M.Com2(2)	10.32
8.	MW6	9.2
9.	MS4	2.7
10.	MS8	11.3
11.	MS9	2.7
12.	MWS2(1)	2.02
13.	MBK2(1)	10.7
14.	MBK2(3)	2.13
15.	Sw/Tw1	3.28
16.	Sw/Tw2	3.0
17.	Sw/Tw3	14.92
18.	Sw/Tw5	3.2
19.	MG6	2.7
20.	MG7	2.23

Table III
Biochemical Characterization of M.Com2(1) isolate

S.No.	Biochemical test	Observation
1.	Gram Staining	Positive
2.	Catalase test	Positive
3.	Oxidase test	Positive
4.	Hydrogen sulfide production test	Positive
5.	Urease test	Negative
6.	Casein hydrolysis	Positive
7.	Gelatin hydrolysis	Positive
8.	Amylase test	Positive
9.	Carbohydrate catabolism	Positive
10.	Indole production test	Negative
11.	Citrate utilization test	Positive

CONCLUSION

The present study was outlined to isolate maximum PHB producing bacterial strain among the screened isolates to optimize its culture parameters so as to obtain highest PHB yield. After the experiments, the PHB production was found to be increased, C:N ratio have a great influence on PHB production. Therefore it can conclude that bacterial PHB production can be improved by optimizing the fermentation conditions at industrial level. In this piece of work, bacterial isolate *Bacillus megaterium* was found to be high PHB producers in optimized culture conditions, showing a capacity for its up gradation at industrial level. Thus, the present study has provided valuable data about the optimized conditions for PHB production that can be utilized at industrial level for PHB production, a fast emerging alternative for petroleum based synthetic plastics.

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