Production and characterization of Poly hydroxy butyrate (PHB) with *Streptomyces olivochromogenes* isolated from Cashew nut shell liquid (CNSL) deposited soil

D. Anoop Jacob, B. Rajagopal, S. Jeeva

Abstract- Cashew nut shell liquid (CNSL), a by-product of the cashew kernel industry, is a caustic, viscous, toxic, dark liquid rich in phenolic compounds. In cashew processing unit, the cashew nut shell liquid is the main effluent causing environmental pollution. But it can be considered as a versatile raw material with wide applications in the form of surface coatings, paints and varnishes, as well as the production of polymers. The aim of the study was to isolate, screen and identify potential bacteria for the production of a biodegradable plastic material called poly-β-hydroxybutyrate (PHB). The bacterial isolates from CNSL (41 Nos.) were screened for PHB production by Sudan black B staining. Among the isolates only 6 Showed significant intracellular PHB accumulation and were selected for further studies. Among the isolates PHB2KMD41 showed highest PHB accumulation while screening and was selected for downstream studies. The strain was identified at molecular level as *Streptomyces olivochromogenes*. The 16S rRNA gene sequence from the isolate *Streptomyces olivochromogenes* (PHB2KMD41) was submitted at NCBI GenBank under the accession number MN620387. The optimized conditions for PHB production by the strain are pH 7.5, Carbon source: Starch, C: N 15:1, temperature 30ºC and time 48h. The strain was found to be capable of utilizing starch as a substrate for PHB production which makes the fermentation process cost effective. The PHB produced by the strain was completely biodegradable and biocompatible in nature which depicts its application potential in biomedical industries. The drug conjugation study of the polymer with the antibiotic gentamycin proved its potential to use in drug delivery system. The identity of the polymer was analyzed by FT-IR and confirmed. The PHB production with natural starch extracted from food waste was also successful which reveals the industrial potential of the strain. All these results are promising which marks the biotechnological importance of the strain isolated from CNSL deposited soil.

Keywords: Biocompatible, Biodegradable, Bioplastics, Cashew nut shell liquid (CNSL), FT-IR, Poly hydroxy butyrate (PHB), *Streptomyces olivochromogenes*

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1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) intracellular inclusions seen in many bacteria are reserves of carbon and energy (1). They are synthesized and deposited when bacterial cells are cultured in a medium containing surplus amount of carbon source with inadequate supply of other nutrients (1).

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These are biodegradable-biocompatible thermoplastics, non-toxic, hydrophobic, impermeable to gases, piezoelectric, enantiomerically pure and show a high degree of polymerization with molecular weights of 20,000 to 30 million Daltons (2–4). Polyhydroxybutyrate (PHB), a lipid-like polymer of 3-hydroxybutyrate, is a representative member of polyhydroxyalkanoates (PHAs) formed in many bacteria. Cashew nut shell liquid (CNSL), a by-product of the cashew kernel industry, is a caustic, viscous, toxic, dark liquid rich in phenolic compounds. In cashew processing unit, the cashew nut shell liquid is the main effluent causing environmental pollution. But it can be considered as a versatile raw material with wide applications in the form of surface coatings, paints and varnishes, as well as the production of polymers. PHAs have attracted a great deal of attention because of their bio-degradability and thermoplastic properties (5). They show physical and material properties which make them suitable for

applications in various fields such as manufacturing of packaging materials, biomedical implant materials, drug delivery carriers, biofuels, water resistant coatings on cardboard or paper, additives in cosmetics and in food processing industries (6–8). PHAs can be produced from renewable resources and they are considered as an alternative biodegradable plastic produced from fossil oils (9). Commercial production of PHA is limited by the high cost of production compared to conventional plastics. The main focus on the biopolymer research is to develop economically feasible methods for the large-scale production of good quality biopolymer. This study was focused on the polyhydroxy butyrate (PHB) accumulation property of *Streptomyces olivochromogenes* isolated from CNSL deposited soil.

2 MATERIALS AND METHODS

2.1 Sampling, isolation and maintenance of bacterial strains

CNSL deposited soil samples were collected in sterile bags from cashew industries across Kanyakumari district, Tamil Nādu, India and brought to the laboratory in sterile conditions. Bacterial strains were isolated on nutrient agar medium (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract, 1.5 g of yeast extract and 15 g of agar per liter at pH 7.4) by serial dilution method and incubated at room temperature (30°C) for 48 h. Based on colony morphology and pigmentation bacterial isolates were selected and pure cultures were prepared and were maintained on nutrient agar slants and stored at 4°C (10, 11).

Fig 1 CNSL Deposited Soil

2.2 Preparation of seed inoculum

One loop full of the culture from slant was inoculated in 5 mL of sterile nutrient broth (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract and 1.5 g of yeast extract per liter at pH 7.4). After incubation for 24 h at room temperature, 1% (v/v) of culture having 10^8cells/mL was aseptically transferred into 100 mL sterile nutrient broth and incubated for 18 h at room temperature. From this, inoculum was added at 1% level in all the polymer quantification experiments.

2.3 Screening of isolates for PHB production

Initially the PHB producing strains were screened by direct microscopy method. The isolated bacterial strains were grown on PHB production media - half strength nutrient agar (2.5 g of peptone, 2.5 g of sodium chloride, 0.75 g of beef extract, 0.75 g of yeast extract and 15 g agar per liter at pH 7.4 supplemented with 20 g/L glucose, after 48 h of incubation at room temperature (12). The bacterial colonies on Petri plates were stained with Sudan Black B solution (0.03% in ethanol) and kept undisturbed for 30 min. The excess stain was washed out by sterile saline and the dark blue colored inclusion bodies were identified under light microscopy in oil immersion. The PHB producing strains screened were further confirmed by fluorescent microscopy (13).

2.4 Staining for PHB accumulation by fluorescent microscopy

48 h old bacterial cultures grown in basal medium (1.5 g of peptone, 1.5 g of yeast extract, 1 g of Na_2HPO_4 and 0.2 g of $MgSO_4 \bullet 7H_2O$ per liter, pH 7.2) supplemented with 20 g/L glucose at room temperature were taken for staining and microscopic analysis. Nile Red stained cell suspension was taken on a glass slide and was covered by a cover slip. The cells were imaged with a fluorescent microscope with 50× magnification and excitation at 561 nm (14-16).

2.5 Evaluation of bacterial strains for PHB production

From the isolates, strains producing considerable amount of PHB granules were inoculated in 1000 mL basal medium with 20 g/L of glucose and incubated at room temperature for 48 h at agitation rate of 150 rpm. The cells were harvested and washed with sterile normal saline. The biomass obtained was dried overnight and the cell dry mass (CDM) was calculated. Polymer was extracted from the dried cells, weighed and estimated the yield in percentage (w/w) (17, 18).

2.6 PHB extraction by sodium hypochlorite digestion

The extraction method developed by Shi et al (1997) is the commonly used extraction method for PHB from bacterial cells and has been used in this work also. Cultures were centrifuged to get rid of the unused medium. The resulting cell pellet was dried overnight and the cell dry weight was measured. The dried pellet was incubated in sodium hypochlorite solution for an hour at 65°C for PHB extraction and then centrifuged again to remove the digested biomass as supernatant from the extracted PHB which sediment at the bottom. The crude PHB which was extracted was washed successively by centrifugation in distilled water, acetone and methanol and air dried before proceeding

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to the quantification and the percentage PHB yield (w/w) was calculated (19).

2.7 PHB quantification by Crotonic acid method

The extracted PHB was dissolved in10 mL concentrated H_2SO_4 and the tube was sealed with a glass stopper. This tube was heated in a boiling water bath for 10 minutes to complete the conversion of PHB into crotonic acid. The sample was cooled and vortexed well and transferred to a silica cuvette for measurement of UV absorbance in a spectrophotometer. Commercial PHB (Sigma Aldrich®) was taken and converted to crotonic acid by similar method (19). The UV absorbance from this standard PHB was used to construct a standard curve and the concentration of the PHB extracted from the culture was determined with the help of this standard curve.

2.8 Morphological, biochemical and molecular characterization

The isolate was morphologically and biochemically characterized by following the standard microbiological methods (20, 21).

Gram Staining, Motility test, Starch hydrolysis, Cellulose hydrolysis, Gelatin hydrolysis, Casein hydrolysis, Methyl Red test, Voges-Proskauer test, Catalase test, Indole test, Sucrose fermentation test, Glucose fermentation test, Fructose fermentation test, Lactose fermentation test, Galactose fermentation test and Mannose fermentation test

2.9 Molecular identification

For molecular characterization, genomic DNA was isolated from the strain by Phenol - Chloroform method (22). 16S rRNA gene was amplified using the universal eubacterial primers 27F and 1492R (23). The PCR cycling condition used are as follows: 35 cycles: 94°C for 30 seconds, followed by annealing at 54°C for 45 seconds, and then by extension at 72°C for 45 seconds. After 35 cycles, the profile was linked on hold at 4°C. The PCR amplicon were visualized using Agarose gel electrophoresis. Sequencing PCR was done with ABI PRISM Big Dye terminator v3.1 cycle sequencing kit and the sequencing was done in AB 3730 DNA analyzer (Life Technologies, CA, USA). The sequences obtained were viewed with ABI sequence scanner v1.0, compiled and aligned using BioEdit version 7.0.9.0. (24).

2.10 Analysis of PHB production with different Carbon sources, Temperature, C/N Ratio, and pH

The PHB accumulation property of the isolate was evaluated using different carbon sources for 48 h at agitation rate of 150 rpm. 20 g/L of glucose, sucrose, fructose, maltose and starch were supplemented independently as carbon sources in 1000 mL basal medium (half strength nutrient broth). After incubation, the biomass was harvested; the polymer was extracted using sodium hypochlorite chloroform extraction method and quantified using UV visible spectrophotometer by converting PHB to crotonic acid using concentrated sulphuric acid.

The PHB accumulation property of the isolate to different temperatures was observed at 30°C, 35 ^{O}C, 40 ^{O}C and 45 ^{O}C in the basal medium for 48 h at agitation rate of 150 rpm. After incubation, the biomass was harvested, the polymer was extracted using the same methods described before and results were compared.

The PHB accumulation property of the isolate to utilize different C/N ratio (Starch/peptone) was analyzed with 5:1, 10:1, 15:1, 20:1 in the basal medium at 37^{0}C for 48 h at agitation rate of 150 rpm. After incubation the polymer was extracted and quantified as mentioned above.

The PHB production capacity of the isolate was observed at different pH 6, 7, 7.5,8 etc. After incubation at 37°Cfor 48 h with an agitation rate of 150 rpm the polymer was extracted and quantified as described earlier.

2.11 Time-course analysis of PHB production

Polymer accumulation rate of the isolate was studied at different time points and were estimated by culturing in 1000 mL basal medium supplemented with 20 g/L glucose at 37^{0}C and agitation rate of 150 rpm. The cells were harvested at each 6 h interval up to 72 h and the PHB content were estimated as previously mentioned.

2.12 Polymer Characterization by FT-IR

The PHB extracted from the organism was analyzed by FT-IR spectroscopy. It was used under the following conditions: spectral range, 4000-400 cm^{-1} to confirm the functional groups of the extracted polymer.

2.13 Biocompatibility studies of PHB

Cytotoxicity effects of PHB polymer sheets were investigated on cultured Chang liver cells by MTT assay. 100 µl of appropriately diluted cells were seeded into each well

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of the 96 well plates. The plates were incubated for 24 hrs. Extracted and purified PHB from the bacterial isolate was surface sterilized with 70% ethanol and inoculated into the wells (20 mg/well). The plates were incubated for 24 h at 37°C in CO_2 incubator. After incubation the media was discarded from 96 wells and carefully aspirated using pipette. 50 µL of serum-free media and 50 µL of MTT (1 mg/ml) solution was added into each well. The plates were incubated at 37°C for 1-3 hours. After incubation, 150 µL of MTT solvent (100% DMSO) was added into each well. The plates are kept at 37°C 10 minutes. Occasionally, pipetting of the liquid was done to fully dissolve the MTT formazan. The absorbance was read at 570 nm, within 1 hour. The percentage of cell viability was calculated by comparing with the control.

$$Percentage\ cell\ viability = \frac{Average\ Absorbance\ of\ test}{Average\ Absorbance\ of\ Control} \times 100$$

2.14 PHB drug encapsulation

10 mg of PHB was dissolved in 5 mL of boiling chloroform, and cooled to room temperature; 40 mg/mL of gentamycin was mixed and stirred for 10 min. After stirring, the homogenous slurry thus obtained was collected, air dried and the drug conjugated PHB polymer was used for antibacterial studies against E. *coli* and S. *aureus.*

2.15 PHB degradation study

The degradation of PHB was studied by taking a known weight of PHB sheet. It was then kept in soil slurry and the degradation proceedings were observed. Each week the sheet was taken out washed with sterile distilled water to remove any soil residual particles and was left to dry at room temperature.

The weights of the samples were recorded and the degradation percentage was calculated as a function of weight loss using the equation given below (25).

Degradation % = [(W1 - W2)/W1]100

where W1 is the initial weight of the film and W2 is the weight of the film after degradation.

2.16 PHB production using food waste

Two hundred grams of banana and tapioca peels of each were washed thoroughly, peeled, sliced, and chopped into small chunks. The distilled water was added and the extraction process was carried out through the use of a centrifuge at different speeds (1000, 2000, 4000) rpm for different periods of time (5, 10, 15 min). Thereafter, the centrifuged samples were filtered using Whatman no.1 and the supernatant was neglected to obtain wet starch. The wet starch was dried at room temperature for 5 h, then crushed into a fine powder and used as a substrate for PHB production.

2.17 NCBI GenBank Submission

The 16S rRNA gene sequence from the isolate *Streptomyces olivochromogenes* (PHB2KMD41) which showed highest potential to produce PHB was submitted at NCBI GenBank under the accession number MN620387.

3 Results and Discussion

3.1 Isolation and screening of PHB producing isolates

From the CNSL sample collected, large number of morphologically different colonies was isolated on the nutrient agar plates by serial dilution spread plate method (Fig 2). Based on colony morphology and characteristics 41 colonies were selected for downstream studies. Based on microscopy staining results six isolates were found to be PHB accumulating (Fig 3) and were selected for downstream studies.

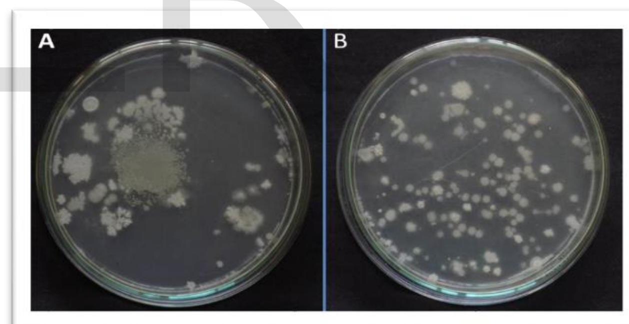

Fig 2: Serial dilution plates; A- 10^{-4} dilution; B- 10^{-3} dilution

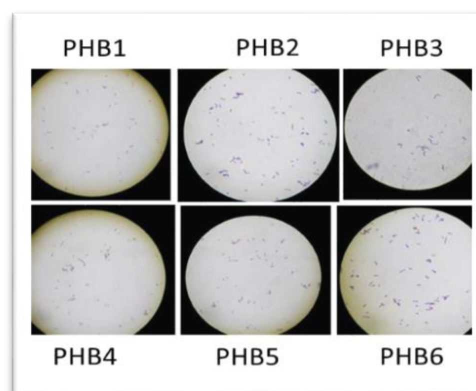

Fig 3: PHB screening – Sudan black B staining

3.2 Evaluation of bacterial strains for PHB production

For quantification of PHB produced by different bacterial strains isolated crotonic acid method was used. A standard crotonic acid curve was generated (Fig 4). The PHB producing capacity of different bacterial strains isolated

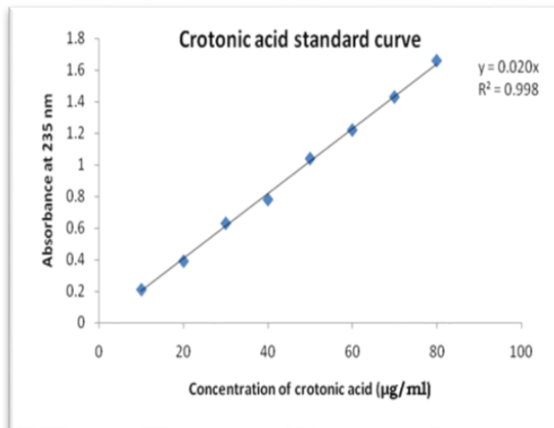

Fig 4: Crotonic acid standard curve for PHB quantification

were depicted in (table 1). The strain PHB 2 was found to be the most potent one and the downstream work was done in it.

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3.3 Strain identification of PHB2KMD41

Morphological and biochemical characterization of the isolate PHB 2 is given in the table 2.

Table 2: Morphological and biochemical characterization of the strain PHB2KMD41

Morphological:	
Shape	rods
Gram stain	positive
Motility	motile
Biochemical tests:	
Catalase	negative
Voges- Proskauer	positive
Indole production	negative
Methyl red	negative
Hydrolysis of:	
Casein	positive
Gelatin	negative
Starch	positive
Cellulose	negative
Carbohydrate's fermentation:	
Glucose	positive
Fructose	positive
Sucrose	positive
Lactose	positive
Galactose	positive
Mannose	positive

The strain PHB 2 was found to be starch utilizing which depicts its potential to use agricultural waste as a substrate for PHB production. The PHB production by the strain was further confirmed by fluorescent microscopy by

Table 1 PHB production with different bacterial strains

Strains	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield (w/w)
PHB1	0.46	1.02	51	0.051	11.08
PHB2KMD41	0.091	1.63	81.5	0.082	**90.1**
PHB3	0.028	0.24	12	0.012	42.85
PHB4	0.055	0.79	39.5	0.04	72.72
PHB5	0.44	0.98	49	0.049	11.13
PHB6	0.032	0.35	17.5	0.018	56.25

Nile red staining. The PHB granules will be stained as red as shown in fig 5. So, the strain was confirmed as PHB positive.

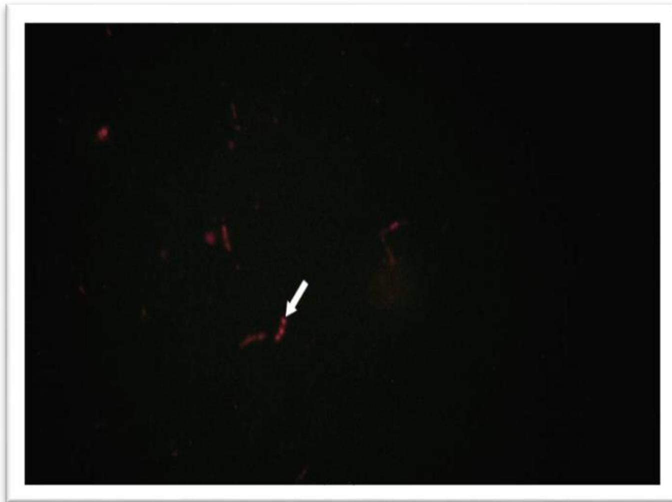

Fig 5: PHB Nile red staining

3.4 Molecular identification of the strain PHB2KMD41

The genomic DNA from the strain PHB 2 was isolated using phenol chloroform isoamyl alcohol extraction method. The genomic DNA integrity was checked by agarose gel electrophoresis (Fig 6).

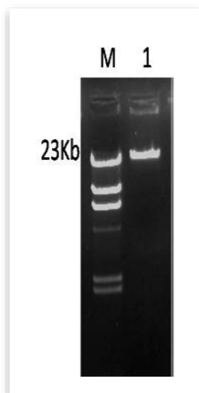

Fig 6: Genomic DNA isolated from strain PHB2KMD41; M HindIII λ DNA ladder (Fermentas), Lane 1- Genomic DNA isolated from PHB2KMD41

A260/A280 was found to be 1.91 (1.8 for pure DNA) and was found to be compatible for downstream molecular biology works. The 16S rRNA gene was amplified successfully from the DNA (Fig 7) and sequencing was done for the strain identification.

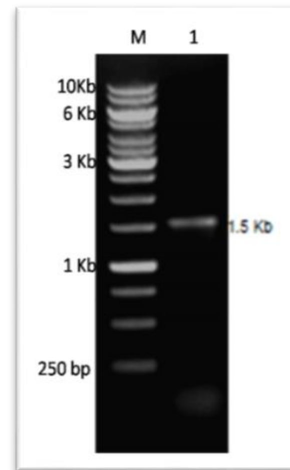

Fig 7: 16S rRNA gene amplified from the strain PHB2KMD41; Lane M 1kb DNA ladder (Fermentas), Lane 1 16S rRNA gene

The strain was identified as *Streptomyces olivochromogenes*, which is a known PHB producing strain. The BLAST result of the sequence was depicted in figure 8.

Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Streptomyces olivochromogenes strain ICTA44 16S ribosomal RNA gene, partial sequence	2442	2442	100%	0.0	99.63%	KF372430.1
Streptomyces sp. APL1 16S ribosomal RNA gene, partial sequence	2388	2388	100%	0.0	98.95%	JN387596.1
Streptomyces antibioticus strain HBUM174911 16S ribosomal RNA gene, partial sequence	2388	2388	100%	0.0	98.95%	EU841627.1
Streptomyces antibioticus gene for 16S rRNA, partial sequence, strain: NBRC 12652	2388	2388	100%	0.0	98.95%	AB184108.1
Streptomyces olivochromogenes gene for 16S rRNA, partial sequence, strain: NBRC 12444	2388	2388	100%	0.0	98.95%	AB184097.1
Streptomyces sp. APL2 16S ribosomal RNA gene, partial sequence	2386	2386	99%	0.0	98.95%	JN387597.1
Streptomyces lavenduligriseus strain NBRC 13405 16S ribosomal RNA, partial sequence	2383	2383	100%	0.0	98.88%	NR_112391.1
Streptomyces sp. 16S rRNA gene, partial, strain NT358(K7)	2379	2379	100%	0.0	98.80%	AJ002085.1
Streptomyces eurythermus strain ATCC 14975 16S ribosomal RNA, complete sequence	2366	2366	100%	0.0	98.65%	NR_025869.2
Streptomyces sp. FLA partial 16S rRNA gene, strain FLA	2366	2366	100%	0.0	98.65%	AM234613.1
Streptomyces eurythermus 16S ribosomal RNA, complete sequence	2366	2366	100%	0.0	98.65%	D63870.1
Streptomyces anisochromogenes 16S ribosomal RNA gene, partial sequence	2362	2362	100%	0.0	98.58%	EU409070.1
Streptomyces lavenduligriseus strain GA1-2 16S ribosomal RNA gene, partial sequence	2359	2359	99%	0.0	98.87%	MG592748.1
Streptomyces sp. APL3 16S ribosomal RNA gene, partial sequence	2357	2357	100%	0.0	98.44%	JN387598.1
Streptomyces eurythermus strain NBRC 12764 16S ribosomal RNA, partial sequence	2353	2353	100%	0.0	98.35%	NR_112265.1
Streptomyces nogalater strain AN24 16S ribosomal RNA gene, partial sequence	2344	2344	100%	0.0	98.35%	KX789179.1
Streptomyces anisochromogenes subsp. anisochromogenes strain NJM12153 16S ribosomal RNA gene, partial sequence	2344	2344	100%	0.0	98.35%	GU350510.1

Fig 8: NCBI BLAST result of 16S rRNA gene from PHB2KMD41

The 16S rRNA gene sequence from the isolate *Streptomyces olivochromogenes* was submitted at NCBI GenBank under the accession number MN620387.

3.5 PHB extraction and sheet production

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Table 3.2 PHB production at different temperature

Temperature	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield
30°C	0.097	1.40	70	0.07	72.16
35°C	[illegible]	[illegible]	[illegible]	[illegible]39	50
40°C	[illegible]	[illegible]	[illegible]	[illegible]35	53.84
45°C	[illegible]	[illegible]	[illegible]	[illegible]22	52.38

Table 3.3 Time Course analysis of PHB production

Time	Cell Dry weight (g/L)	Absorbance at 600 nm	Absorbance at 235nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield
8h	[illegible]	[illegible]	[illegible]	[illegible]14	0.014	51.85
16h	[illegible]	[illegible]	[illegible]	[illegible]8.5	0.019	61.29
24h	[illegible]	[illegible]	[illegible]	[illegible]33	0.033	54.09
32h	[illegible]	[illegible]	[illegible]	[illegible]9.5	0.040	55.55
40h	[illegible]	[illegible]	[illegible]	[illegible]	0.042	53.16
48h	0.081	1.73	0.99	49.5	0.050	61.72
56h	0.061	1.37	0.57	28.5	0.029	47.54
64h	0.052	1.18	0.42	21	0.021	40.38

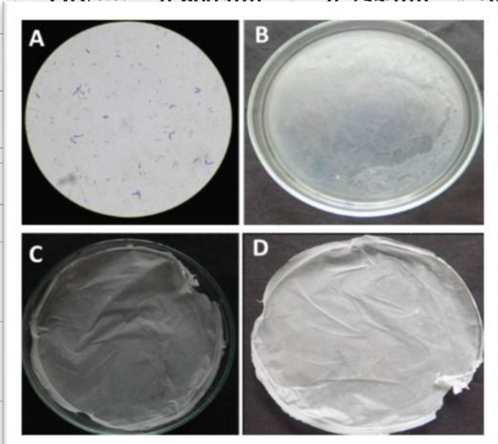

Fig 9: PHB extraction different stages; A- PHB granules inside cell-Sudan black B staining; B- PHB extracted with chloroform; C- Solvent evaporation to form PHB sheet; D- Dry PHB sheet

Table 3 PHB production with different Carbon source

Carbon source	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield (w/w)
Glucose	**0.46**	1.02	51	0.051	11.08
Fructose	0.032	0.35	17.5	0.018	56.25
Maltose	0.028	0.24	12	0.012	42.85
Sucrose	0.055	0.79	39.5	0.040	72.72
Starch	0.091	1.63	81.5	0.082	**90.10**

Table 4: Optimized conditions for PHB production

Carbon Source	Starch
C/N ration	15:1
pH	7.5
Temperature	30 °C
Time	48 h

Table 3.1 PHB production at different pH

pH	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield
6	0.055	0.75	37.5	0.038	69.09
7	0.082	0.81	40.5	0.041	50
7.5	0.09	1.33	66.5	0.067	**74.44**
8	0.064	0.73	36.5	0.037	57.81

The selected isolate was screened for PHB production. The production of PHB granule was confirmed by Sudan Black staining. After 48 h of incubation, it was followed by extraction using chloroform. A thin PHB sheet was formed after chloroform was completely vaporized. Different stages of PHB polymer extraction and sheet production are depicted in fig 9.

The PHB sheet produced was used for downstream works such as drug encapsulation and biocompatibility studies.

3.6 PHB production optimization using the strain PHB2KMD41

The PHB production process with PHB2KMD41 was optimized with different carbon sources, pH, temperature, incubation time and different starch/peptone concentrations. The results were summarized in table 3,3.1,3.2,3.3.4 and represented in fig 10-14. The optimized conditions for, PHB production by the strain is given in table 4.

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Table 3.4 PHB production with different C/N ratio (Starch/peptone)

C/N ratio	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (μg/ml)	PHB yield (g/L)	Percentage PHB yield
5:1	0.063	0.73	36.5	0.037	58.73
10:1	0.079	0.86	43	0.043	54.43
15:1	0.095	1.43	71.5	0.072	**75.78**
20:1	0.077	0.69	34.5	0.035	45.45

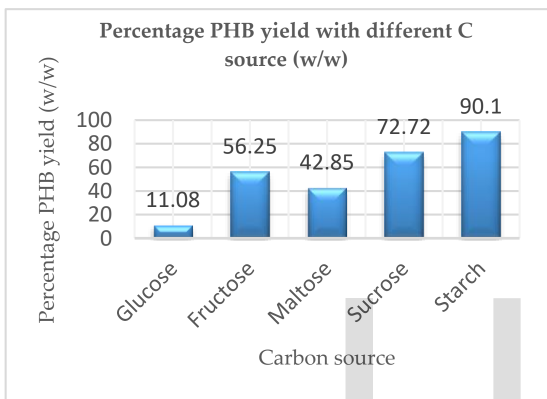

Fig 10: PHB production at different Carbon Source by PHB2KMD41

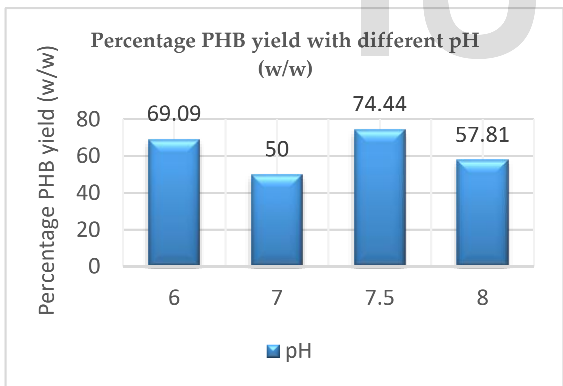

Fig 11: PHB production at different pH by PHB2KMD41

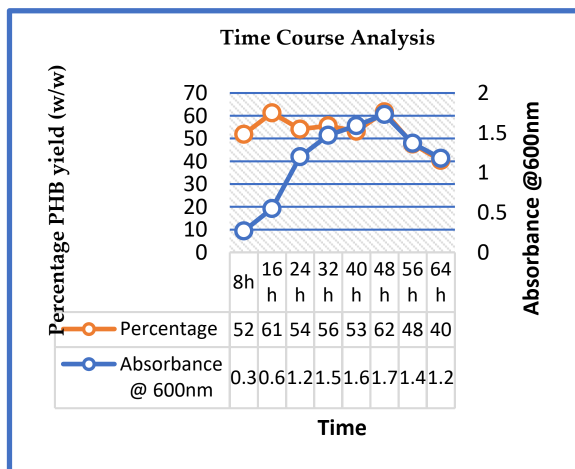

Fig 12: Time Course Analysis of PHB2KMD41

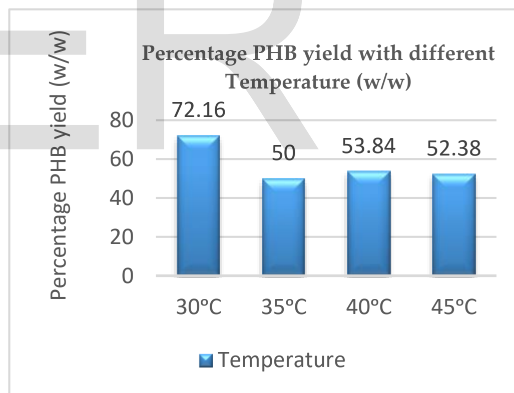

Fig 13: PHB production at different temperature by PHB2KMD41

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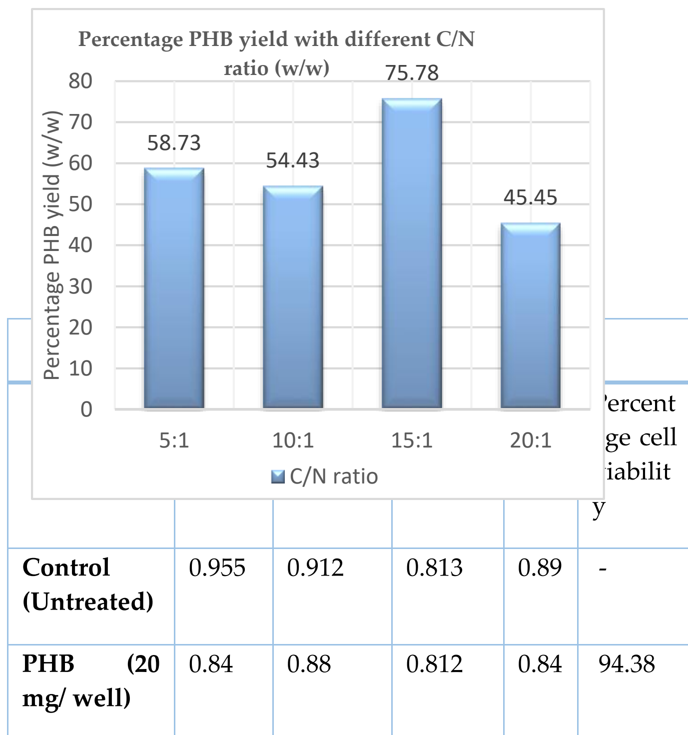


					Percentage cell viability
Control (Untreated)	0.955	0.912	0.813	0.89	-
PHB (20 mg/ well)	0.84	0.88	0.812	0.84	94.38

Fig 14: PHB production with different C/N ratio by PHB2KMD41

3.7 Antibacterial study

The inhibitory effect of PHB films incorporated with gentamycin was tested against bacterial strains using agar diffusion assay (Fig 15). The antimicrobial activity of phb+gentamycin against bacteria is dependent on the time of exposure, concentration, and target organism. The results were summarized in table 6.

Table 6: Antimicrobial activity screening of PHB film encapsulated with antibiotic gentamycin

Organism	**Diameter of the zone (mm)**	
	Control (Gentamycin) 80 mcg	Sample (PHB+Gentamycin)
S. aureus	34	35
E. coli	30	32

The drug encapsulation results are promising, revealing its potential to be developed as a drug delivery system.

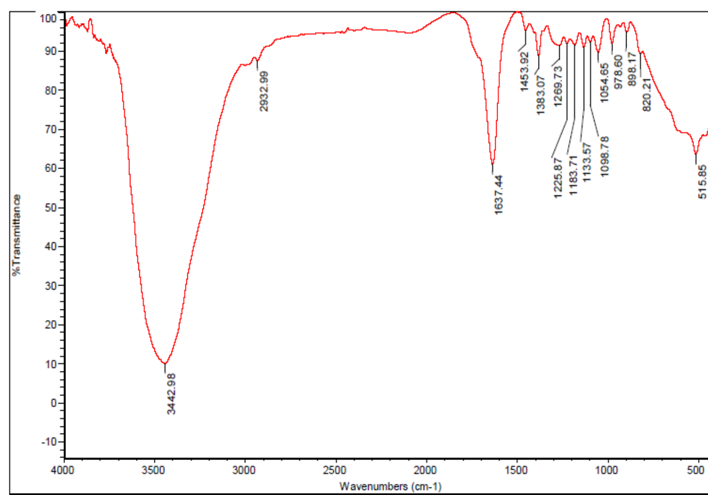
3.8 PHB biocompatibility study in Chang liver cell line

The word biocompatibility refers to the interaction of a living system or tissue or cells with a finished medical device or component materials. In the simplest sense, a biocompatible material or device does not harm the patient. Cytotoxicity is an in vitro biocompatibility test performed on mammalian cells in culture. In order to check the biocompatibility of PHB sheets produced by the strain PHB 2 MTT assay was performed in normal liver cell line (Chang liver). The results were summarized in table 7.

The PHB sheets produced did not showed significant cytotoxicity towards the normal liver cell lines. It indicates its potential in biomedical applications (26-28).

3.9 Polymer characterization by FT-IR

The characteristic peaks mentioned in the table 8 and figure 16, 17 was identified in the FTIR analysis of the PHB



extracted from the strain PHB2KMD41. These peaks confirm that the identified polymer is PHB (29-31).

Table 9: PHB degradation in soil slurry		
Figure Name	Number of days	Weight of PHB sheet(mg)
A	1	20
B	4	17
C	8	14
D	12	11
E	16	4
F	20	2

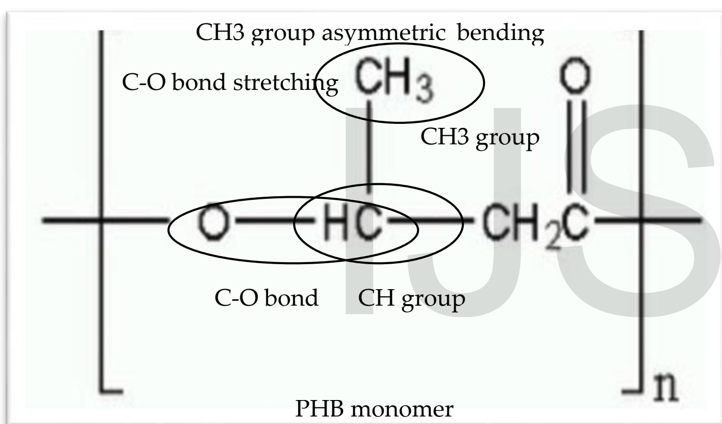


Fig 17: PHB monomer – Groups identified by FT-IR

Table 8 Comparison of FT-IR analysis			
Sl no	**Synthesized PHB**	**Standard PHB**	**Group**
1	3442	3424	OH group
2	1000-1300	1000-1300	Stretching of C-O bond
3	2931	2927	CH3 group
4	1453	1452	asymmetric bending of CH3 group
5	1269	1276-82	CH group
6	1637	1639	thioester C=O valence

3.10 PHB biodegradation analysis

The degradation of PHB was studied by weighing the dried PHB sheet at an interval of about 4 days. The results were summarized in table 8 and fig 18, 19. The PHB produced by the strain was found to be fully biodegradable within 1 month in soil slurry. The Results are summarized in table 9.

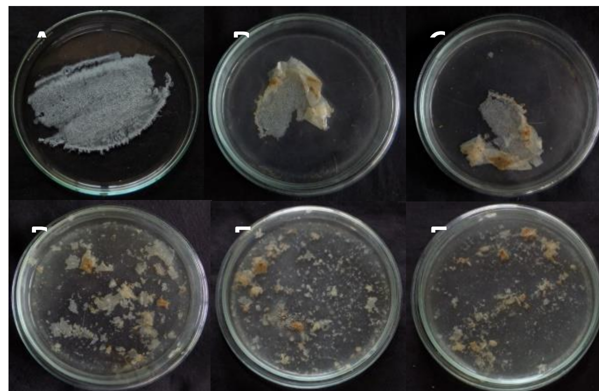


Fig 18: PHB biodegradation in soil slurry

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3.11 PHB production with starch extracted from food waste

The bacterial strain *Streptomyces olivochromogenes* (GenBank Accession No: MN620387) isolated from CNSL is a good candidate for commercial production PHB. The strain was identified at molecular level by 16S rRNA gene sequencing. The biochemical analysis of the strain revealed its potential to produce extracellular enzymes like amylase and cellulose. This will enable the strain to utilize cheap substrates like food and agricultural waste for the economic production of PHB. In order to access this, PHB production by the strain with starch extracted from food waste was carried out. The PHB production was visualized by Sudan

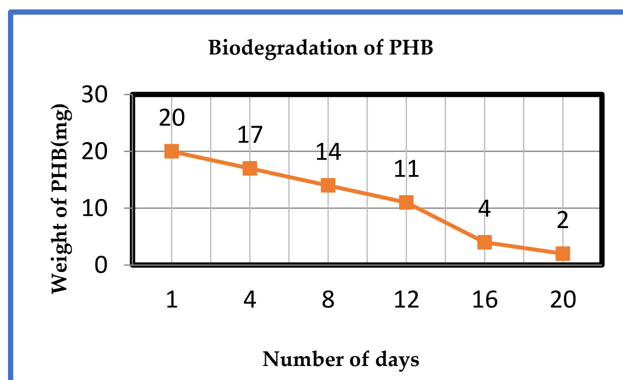

Fig 19: Rate of PHB biodegradation in soil slurry

Black B ed in table 10.

Fig 20: PHB Sudan Black B staining

4 Conclusion

The present study focused on the isolation and screening of PHB producing bacteria from CNSL deposited soil. Six PHB producers were isolated and among them the potent strain PHB2KMD41 was screened out. The strain was then identified by standard molecular and biochemical techniques. This strain was then used for mass production of PHB and its extraction was carried out. The polymer was identified as PHB by FT-IR analysis. Its biocompatibility and drug encapsulation properties were analyzed. The PHB was found to be fully biodegradable. The strain also proved its potential to use food and agricultural waste as substrate for PHB production. All these results are promising especially the strain can utilize starch as a substrate which makes the fermentation process cheaper. The current study is a key for the optimized PHB polymer production using cheap carbon sources for the further reduction in production cost and for large scale production.

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Table 10: PHB production with starch extracted from food waste

Starch from Tapioca/Banana peel	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield
1:1	0.048	0.77	38.5	0.039	81.25

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