International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 492-502
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Production and Characterization of Bioplastics from Marine Bacterium *Bacillus Cereus*

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Received: 8 Oct 2018/ Accepted: 10 Nov 2018/ Published online: 01Jan 2019
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Abstract

Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic. A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. The aim of the present investigation was production and optimization of bioplastic by using the marine bacterium *Bacillus cereus.* Mangrove soil samples was collected from Vellar estuary and serially diluted. The diluted samples were inoculated in the surface of Mineral agar medium by spread plate technique. Totally 16 different strains were isolated from that 10strains showed the accumulation of PHB in the nitrogen deficient medium. Among which *Bacillus cereus* showed the maximum PHB accumulation. In the present study temperature showed the profound influence on the PHB production and optimum temperature for maximum production was found to be 35°C. pH also showed influenced on the growth and the optimum pH-7 for PHB production. Salinity-0.5% was optimum for PHB production. In the present study, sucrose and peptone was found as the best carbon and nitrogen source for maximum growth. Bacillus cereus can be used in industries for large scale production and also alternate to produce truly biodegradable polymers.

Keywords

Bioplastics, *Bacillus cereus*, PCR and FTIR.

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INTRODUCTION

Plastic materials which have made entry in every field of human life are now causing serious environmental problems due to their non biodegradability. The natural qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials. One alternative is to produce truly biodegradable polymers, which may be used in the same applications as the existing

synthetic polymers. These materials, however, must be processible, impervious to water and retain their integrity during normal use but readily degradable in a biologically rich environment.

A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. A plastic produced from PHAs is more ductile and less elastic than other plastics; it is fully biodegradable in both aerobic and anaerobic environments [1]. PHAs are composed mainly of poly-beta hydroxyl butyric acid (PHB) and poly-beta hydroxyl valeric acid (PHV), although other forms are possible. More than 80 different forms of PHAs have been detected in bacteria [2].

A number of bacteria such as *Azotobacter, Bacillus, Archaebacteria, Methylobacteria and Pseudomonas* have been found to synthesize PHA to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) has been the subject of much published research work because it can accumulate PHAs up to 80 per cent dry weight [2].

Biopolymers are one product that can help to overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic [3]. They can be produced by biological systems (microorganisms, plants and animals) or chemically synthesized from biological materials (sugars, starch, natural fats and oils, etc.) [3].

Polyhydroxy alkanoates (PHA) are a good substitute for plastics and elastomers. The PHAs have characteristics similar to petrochemical plastics but are biodegradable [4, 5]. PHAs are polyesters, which are accumulated as energy and/or carbon storage materials by numerous microorganisms, usually when a nutritional component such as nitrogen, phosphorus, sulfur, oxygen, and magnesium is limited in the presence of an excess carbon source [4, 2, 6, 7]. At present, they are produced by microbial fermentation; in the future, production will also be possible by in vitro methods or by agriculture using transgenic plants.

The physical properties of the homopolymer of PHB are similar to those of polypropylene (melting point, crystallinity, glass transisition and temperatures), and represents a stiff and brittle material. They can be used in a wide variety of products including containers, bottles, razors and food packaging materials.

Though several organisms that accumulate PHA have been identified, commercial production of this polymer is limited. Several factors influence the economics of biodegradable polymer production. One main factor is cost of the substrate. The ability to produce biodegradable polymers from inexpensive and renewable carbon sources may improve the economics of the process and lower production cost.

PHAs are natural thermoplastic polyesters, which can be used for manufacture of disposable items such as razors, utensils or different personal hygiene products [2]. They can be used in the manufacture of latex paints [8].

PHAs, being biodegradable and biocompatible, have applications in medical therapeutics. PHAs can be used to fabricate three dimensional, porous, biodegradable heart valve scaffold [9], bone fracture fixation [10], manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents [11]. PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing [2].

MATERIALS AND METHODS

Sample collection and isolation of PHB producing bacteria

Mangrove soil samples were collected from Vellar estuary (Latitude 11 29'N, Longitude 76 46'E), Tamil Nadu. Clean, sterile screw capped bottles were used for sample collection and transport to the laboratory. Soil samples were serially diluted using standard volume (9ml) of sterile distilled water in series 0.1ml of diluted samples were inoculated on the surface of Mineral agar medium by spread plate technique. The plates are incubated at 30^0C for 24hrs. After incubation, individual colonies were selected based on the difference in colony morphology. Pure culture of colonies obtained in Mineral agar medium for PHB screening.

Screening for potential PHB producing species

The presence of PHB granules in bacterial cells was primarily identified by staining with Sudan Black B dye. Specimen was prepared and stained for 10min. After that the slide was immersed and withdrawn several times in xylene and blot dried with an absorbent paper. Finally, the slide was counter stained for 5min. with 0.5% (w/v) aqueous safranin. After drying, the stained slide was examined in a phase contrast microscope for the presence of PHB granules.

Rapid screening for potential PHB producing strain

For rapid screening of PHB producers, nutrient agar medium supplemented with 2 percent glucose was added. The plate was divided into 6 equal parts and in each part, a bacterial isolates were spotted. The plates were incubated at 30^0 C for 24 hours. After incubation, Sudan Black B dye was spread over the plates and allowed for 30 minutes. Plates were washed with ethanol (96%) to remove the excess stain. Based on the dark blue coloured, the colonies were selected for further PHB production.

Fluorescent staining method

In fluorescent staining method 10µl of old culture of the isolate was transferred to an eppendrof tube containing 50µl of acridine orange and incubated for 30 minutes at 30^0C. After incubation, the culture was centrifuged at 4000 rpm for 5 minutes. The pellet was collected and resuspended in distilled water. A smear was prepared on a clean microscopic slide and observed in a fluorescent microscope. The appearance of yellow coloured granules inside indicates PHB production.

Sudan block staining

The positive strains in Sudan black staining was identified according to the method described by Bergey's manual [12] for potential PHB producing strain. The following tests were performed to confirm the species.

Identification of potential strain

Morphological identification

Morphological characteristics of the colony, colour, shape, pigmentation, reverse pigmentation were studied by using the hand lens. Microscopic observations of bacterial strains were studied by preparing the slides and observing under florescence microscope.

Gram staining

Thin smear was prepared from twenty four hour old culture and fixed by gentle heat. The smear was flood with Gram's crystal violet for 1 minute and washed off with water. After that flood with Gram's iodine for 1 minute and decolorized with Gram's decolourizer until no further violet colour comes off. The slide was washed with distilled water and drained. Safranin was applied on smear for 1 minute as counter stain, washed with distilled water and blot dried. The slid was observed under oil immersion objective.

Physiological characterization

Growth at 4^0 C

Twenty four hours grown old culture was spotted on the trypticase soy agar plate and incubated at 4^0 C for 1-2 days. The growth was observed and recorded at the end of the incubation period.

Growth at 41^0 C

Twenty four hours grown old culture was spotted on the trypticase soy agar plate and incubated at 41^0 C for 1-2 days. The growth was observed and recorded at the end of the incubation period.

Spore formation

One to two days old culture was smeared on a glass slides, allow to air dried and heat fixed. The smear was flooded with malachite green and steamed on water bath for 5 minutes. The slide was washed with distilled water and counter stained with safranin for 1 minute. The smear was washed with distilled water, blot dried and placed under oil immersion objective.

Biochemical tests

Biochemical tests were carried out as per the method given by Cappuccino and Sherman [13] with 24 hr old cultures.

Catalase test

The PHB producing organisms was inoculated into nutrient agar slants and were incubated at 30^0 C for 24 hr. After incubation, the tubes were flooded with 1ml of 3% hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was taken as positive for catalase test.

Gelatin liquefaction

The strains were inoculated on gelatin agar tubes and were incubated at 37^0C for 3-6 days. After that, the tubes were placed in refrigerator at 4^0C for15 minutes and observed for gelatin liquefaction.

Starch hydrolysis

The strain were made a single streak on starch agar plate and incubated at 25^0C for 2-3 days. After incubation, the surface of the plate was flooded with Grams iodine solution for 1minute. The plate was examined for the starch hydrolysis around color change of the medium and clear zone surrounding the microbial colonies is a typical positive starch hydrolysis.

Casein hydrolysis

The bacterial strain were spotted on skimmed milk agar plate and incubated at 29^0C for 2 days. The production of halo zone around the colony was taken as positive for the test.

Urease teat

The overnight grown culture was inoculated to the test tubes containing sterilized urea broth and incubated 29^0C for 1-2 days. The expansion of pink shade was taken as positive for the urease test.

Indole production test

The test culture was inoculated into the pre sterilized SIM agar slant and incubated at 30^{0}C for 2 days. After incubation, the tube was added with 10 drops of Kovac's reagent. The production of red colour was taken as positive for the test.

Citrate utilization test

It was done on the Simmon's Citrate Agars slants and a change in the medium colour from green to blue was taken as positive for the test.

Optimization of culture parameters for maximum PHB production

The shake-flask culture of potential strain was further subjected to evaluate the effect of different environmental parameters like temperature (25ºC, 30ºC, 35ºC and 40ºC), salinity (0.0%, 0.5%, 1.0%, 1.5%, and 2.0%) and pH (5, 6, 7, 8, 9 and 10) as well as different carbon (glucose, starch, sucrose and maltose and glycerol) and nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, peptone and beef extract) on cell growth and PHB production.

Mass scale production

The potential strain was cultivated in high sucrose (2%) and low nitrogen (peptone-0.04%) medium with all the optimized parameters in 4 No's of 250ml conical flasks with the optimized culture conditions in a shaker at 150rpm for 60hrs.

Determination of cell concentration

Total cell concentration was determined by weighing the dry cell weight (DCW) obtained as follows. 10 ml culture samples were centrifuged at 12,000 rpm for 15min. at 4ºC. The pellet was resuspended in distilled water (10 ml) and centrifuged again for washing. The washed cells were dried at 105ºC for 1 day in a hot air oven then cooled down. The drying was repeated until constant weight was obtained.

Isolation and purification of PHB granules

Cells containing the polymer were harvested from culture broth by centrifugation at 5,000 rpm for 20min at 4^{o}C washed once with sterile distilled water and suspended in 30% alkaline hypochlorite solution. For one volume of concentrated cells, three volumes of hypochlorite solution was added and digested for 90min. at 37^{o}C. After centrifugation, pellet was dialyzed against distilled water for 24hrs. The pellet was repeatedly washed with distilled water and acetone. This partially purified pellet was dissolved in small volume of chloroform and methanols (2:1 v/v) with the insoluble remain being discarded by centrifugation. The polymer was precipitated from the concentrated solution with 10 volume of ethanol and the resulted material was poured in a pre-weighted glass tray and after complete evaporation, weight of PHB produced was estimated.

FT-IR Analysis

The purified samples were first dried in an oven at 60ºCfor 4hrs. After removing the moisture content; the samples were grown into fine powder. The IR spectrum of the PHB film was recorded with Perkin-Elmer model 297 IR Spectrophotometer. A thin film was scanned between 600 and 4000 wave number 9cm-1 at a speed of 1 micron/min., and with a programmed split opening 2X and air as reference. Infrared spectral analysis of biological material was utilized to investigate their chemical constituents. These are recognized even when the amount of material available is very small.

PRODUCTION USING CHEAPER SOURCES

Mass scale production using cheaper sources

The potential strain was cultivated using sewage water (both diluted and undiluted). To the diluted sewage 1% molasses was added in 1000ml conical flask and kept the optimized culture conditions in a shaker at 150rpm. Biomass estimation and purification of PHB were done as before.

Molecular basis of Bioplastic production (PHA synthase detection)

Template DNA Preparation

Boiling method: This method was used to prepare template DNA from Test bacterial Sample, as well as from negative control bacterial and positive control. The potential strain was incubated overnight at 37ºC on nutrient agar plates. Thereafter, a colony from the plate was suspended in 100µl of sterile distilled water in a 1.5 ml micro-centrifuge tube and boiled for 10 min. The lysate was chilled on ice and then spin at 6000rpm for 5min in a micro-centrifuge to pellet the debris. 2µl of the supernatant was directly used as template in the PCR reactions. The same method was followed for the preparation of template DNA from test bacterial sample.

PCR Amplification

PCR reaction were carried out using the forward primer of 5'AAGGATCCACTACATTCCGCACCAGAATGGG3', and the reverse primer of 5'AACTGCAGTTACTTACTTAGAGCGCTCGTC3'. The PCR cycling conditions were: Initial denaturation at 94ºC for 2 min., denaturation of primer at 94ºC for 1 min., primer annealing at 60ºC for 1 min., and extension at 72ºC for 2 min., for a total of 35 cycles, followed by a 7 min., final extension period. Amplification was performed in a thermocycler.

Detection of PCR products by Agarose Gel Electrophoresis

Agarose gel electrophoresis was done according to the instructions of the manufacturer (Mini Agarose Gel Electrophoresis instrument by Yercaud Biotech, Tamil Nadu, India). The samples were separated at 100V until the tracking dye reach reached 3/4th distance of the gel. The amplified gene product which glows orange to red colour band in the gel was observed and analyzed under the UV-trans illuminator.

Gel Documentation

The gel was photographed; the molecular weight of the product was analyzed by using the gel documentation system (Yercaud Biotech, Tamil Nadu, India). The PCR product size of was determined with the 100 bp DNA ladder in the agarose gel and was verified with the same size of resultant product in the positive control lane. There was no amplification in the negative control lane.

RESULTS AND DISCUSSION

In the present investigation 10 different strains showed the accumulation of PHB in the nitrogen deficient medium isolated from mangrove soil sample collected from Vellar estuary. The isolated strain was biochemically identified as Bacillus cereus (Table 1). Among which *Bacillus cereus* (Fig.1a) showed the maximum PHB accumulation when observed under microscope after staining with sudan black. Juan [14] reported all the isolates were tested for PHB production following the viable colony screening method based on the intensity of staining. The relative occurrence of PHB accumulating microbes from a variety of samples was studied. A striking prevalence was observed in the activated sludge samples of a food processing industry and from mangrove soil samples. Similar results were also observed by Sujatha [15] they obtained higher PHB producers from tannery effluent and sewage sludge samples compared to mangrove soil samples. Substantial research has been carried out PHB production by different species of *Bacillus* and *Pseudomonas* by many workers [16, 17, 18].

Fig 1a: Isolated colonies of *Bacillus cereus*

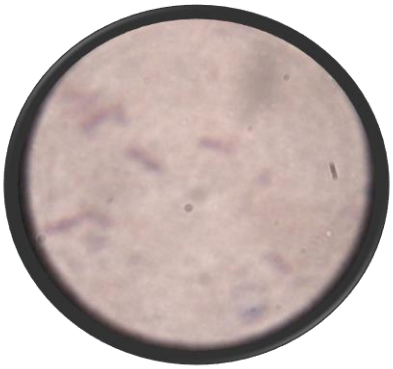

Fig 1b: Microscopic view of PHB granules

Table: 1 Biochemical identification of *Bacillus cereus*

Sl. No.	Morphological tests	Results
1	Colony colour	White
2	Shape	Rod
3	Gram reaction	+
4	Motility	+
5	Fluorescence	-
6	Glucose	A

Sl. No.	Physiological tests	Results
1	Spore formation	+
2	Growth at 4^0C	-
3	Growth at 41^0C	-
4	Xylose	-

Sl. No.	Biochemical tests	Results
1	Catalase test	+
2	Gelatin liquefaction	+
3	Starch hydrolysis	+
4	Casein hydrolysis	-
5	Urease test	+
6	Indole production test	+
7	Citrate utilization	+

In the present investigation presence of PHB granules in bacterial cells were identified by staining with Sudan black and the positive strain was quantified for maximum PHB producing strain was selected for the further experiments (Fig.1b).

Optimization of mass scale conditions has long been used to enhance yields and productivities of many bioprocesses. Hence, in order to maximize PHB production by the selected isolates, various factors such as carbon source, nitrogen source, pH and C:N ratio were optimized. In the present investigation it was observed that PHB concentration was depended on the cell biomass and availability of low nutrient content in the media and other parameters effecting the cell growth.

In the present study mineral medium was used for quantitative analysis of PHB production from *Bacillus cereus* under of varied temperature (25ºC, 30ºC, 35ºC and 40ºC), salinity(0.0%, 0.5%, 1.0%, 1.5%, and 2.0%), pH(5, 6, 7, 8, 9 and 10) and carbon sources (glucose, starch, sucrose and maltose and glycerol), nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, peptone and beef extract). Similarly Choi [19] also studied the biosynthesis of PHB by *Hydrogenophaga pseudoflava* from various carbon sources like glucose, fructose, sucrose, maltose and cellulose (1%) were amended to mineral salts medium and the bacterial isolates grown in them. Glucose was found to be the best carbon source.

In the present study temperature showed the profound influence on the PHB production and optimum temperature for maximum production was found to be 35°C (Fig.2). pH also showed influenced on the growth and the optimum pH-7 for PHB production (Fig.3). Salinity-0.5% was optimum for PHB production (Fig.4). Aslim [20] who observed that the PHB in *Rhizobium* strain grown on yeast extract mannitol broth adjusted to pH 7.0, the amount of PHB in strain was 0.01 to 0.5 g/l and the percentage of PHB in these cells was between 1.38and 40% of cell dry weight. Tavernler [21] also investigated the effect of different nitrogen, carbon and different pH levels on exopolysaccharide and PHB production in two strains of *Rhizobium meliloti.* They reported that these two strains showed higher PHB content at pH 7.0.

In the present study, peptone was found as the best nitrogen source for PHB production and various carbon sources tried and sucrose was found to be best (Fig.5) and the incubation period was 60hrs for maximum growth. Similarly, Sujatha [15] also used LB broth containing glucose 2% as the medium, which favored PHB accumulation due to higher C:N ratio. Working with different carbon sources in MSM broth, Khanna and Srivastav [22] observed higher PHB yield on fructose by *A. eutrophus.* They reported

that glucose and fructose, being monosaccharides were readily utilized by bacteria and, hence, have supported growth and subsequently PHB production. The complex molecules like starch and lactose were not utilized. In the present study also, the isolates did not produce PHB on maltose and starch indicating that the isolates do not possess enzymes involved in the degradation of starch and maltose into glucose. As the complexity of the carbon source increased PHB yield also decreased.

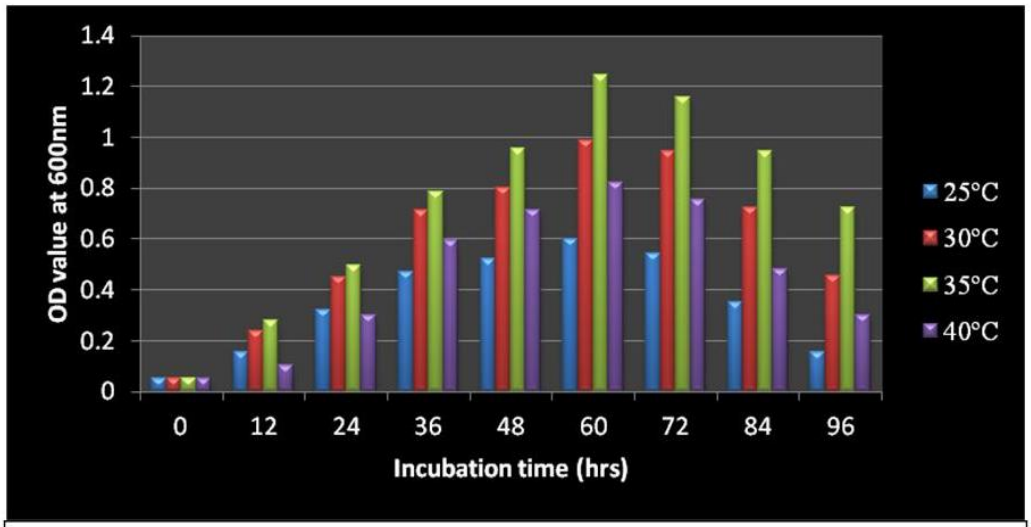

Fig. 2: Effect of Temperature on growth

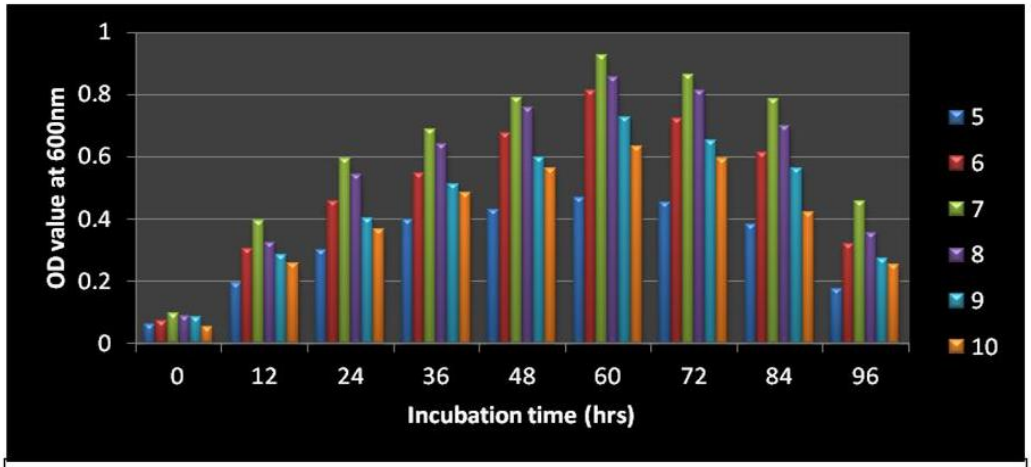

Fig. 3: Effect of pH on growth

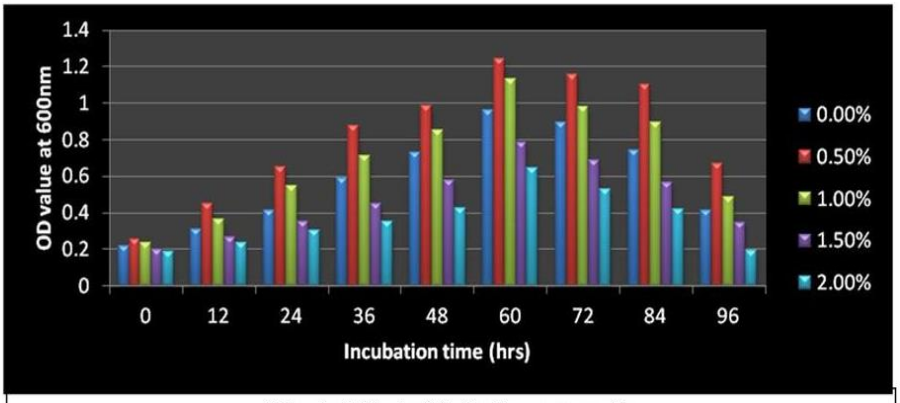

Fig. 4: Effect of Salinity on growth

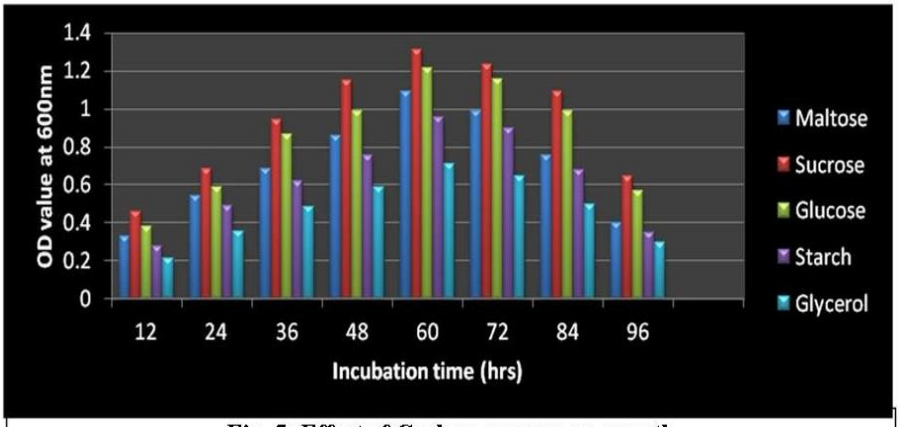

Fig. 5: Effect of Carbon sources on growth

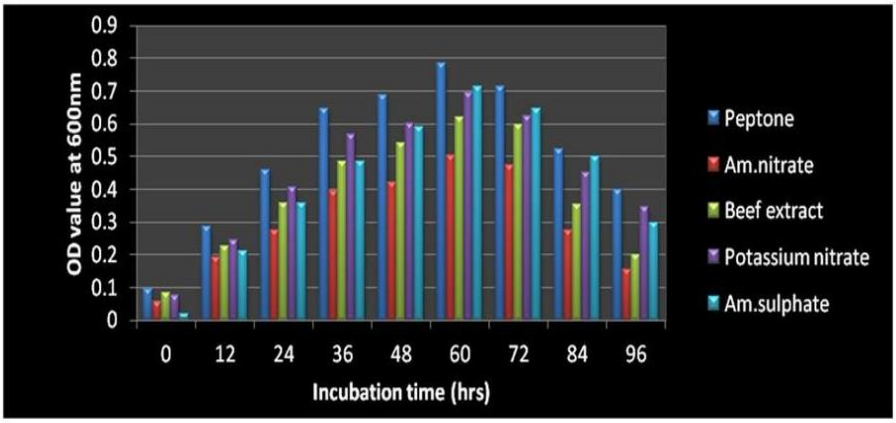

Fig.6: Effect of Nitrogen sources on growth

In the present study nitrogen sources (0.04%) ammonium nitrate, ammonium sulphate, potassium nitrate, peptone and beef extract were optimized among these peptone was best one for specific growth rate of *Bacillus cereus* (Fig.6). Mulchandani , Raje and Srivastava [23,24] also worked on the accumulation of PHB by *A. eutrophus* with different salts of ammonium. The highest PHB was obtained in ammonium sulphate followed by ammonium chloride. The effectiveness of ammonium sulphate at 1g/l in enhancing PHB production is in accordance with the Khanna and Srivastav [22] who found that this level produced the highest PHB by *R. eutropha*. Belfors [25] reported that glucose and ammonium ions were inhibitory at certain levels, which affect the specific growth rate and PHB production. Inhibition by ammonium ions [26] and substrate inhibition by carbon source [27] on PHB production have been reported.

In the present study among different carbon sources tried PHB content was more when sucrose was used as the sole carbon source. On dry weight basis 15g/L accumulation of PHB was noticed. For the production of PHB by using cheaper sources sewage water was tried both as diluted as well as undiluted sample. When sewage water used as such i.e., without any dilution, the PHB production was found to be lesser i.e., only 2.0g/L was obtained. Contrary to this when sewage was diluted and supplemented with 1% molasses the PHB production increased to 19g/L was observed (Table 2). Similarly Sureshkumar [28] proposed the production of PHB from activated sludge of waste water treatment plant of food processing industry waste water. They suggested that production and recovery of PHB from activated sludge could significantly reduce the cost of PHB and at the same time reduce the quality of excess sludge produced in waste water treatment system that required further treatment.

In the present study PCR done with PHA synthase gene specific primers revealed a product of 1000bp confirming the presence of the gene in the strain used. The present study tested seems to have genetic potential for PHB synthesis (Fig: 11).

Table: 2Effect of culture media composition on growth and PHB production by *Bacillus cereus*

Culture	Growth (g/L)	PHB(g/L)	% of yield
Synthetic	20	15	75.0%
Sewage water (undiluted)	5	2.0	40.0%
Sewage water with 1% molasses	26	19	73.07%

Fig.7: Mass cultivation in shaking flask

Fig.8: Mass cultivation in Static Condition

Fig.9: Purification using Dialysis

Fig.10: BioPolymer

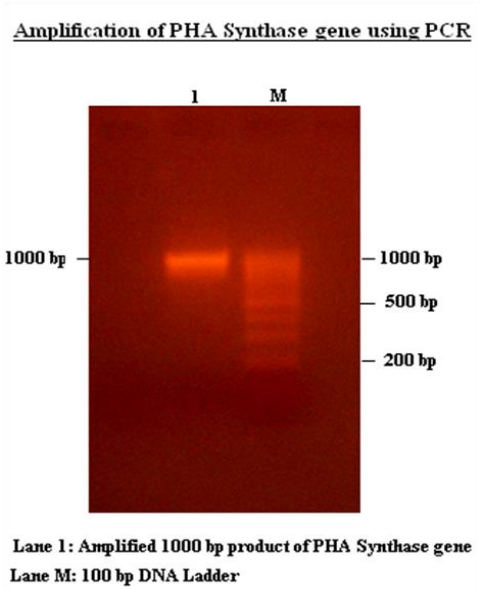

Fig 11: PCR amplification product of PHA synthase gene

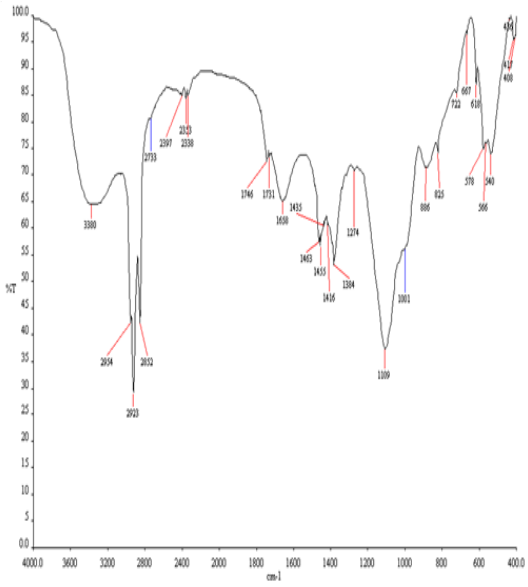

Fig: 12 FT-IR analysis of PHB

The IR analysis showed the presence of CH2- CH3- C=0 methyl esters in the extract. The IR spectrum was conformed to other published reports and thus confirmed as PHB (Fig: 12).

CONCLUSION

The present study not only gave a solution for PHB production but also a permanent solution for converting the unlimited sewage burden in to valuable bioplastics. Thus the present study revealed the possibility for the cheapest production of bioplastics. Hence, in this investigation, attempts were made to isolate PHB accumulating bacteria from mangrove soil and to select the efficient strain *Bacillus cereus*. The process parameters for maximum PHB production were also optimized and scale up using sewage water as a cheaper substrate.

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