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PHB PRODUCTION BY *BACILLUS* SPECIES USING THE CHEAP SUBSTRATE GROUNDNUT OIL CAKE

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ABSTRACT

The present study encomparies of the isolation and production of PHB (Poly-β-hydroxy-butyrate) producing *Bacillus* sp., from soil samples. The accumulation of PHB as granules in the organism was analyzed by Sudan black staining method. The PHB production was determined from the selected strains (B6, B7, B8, B10 and B11) by crotonic acid extraction method using U-V spectrophotometer at 235nm. The optimization of pH and temperature were determined by standard methods. Maximum amount of PHB were obtained by standardizing the physical parameters such as temperature at 37˚C and pH at 7. The cheap substrate Groundnut oil cake was used as a carbon source. The substrate Groundnut oil cake optimization at different concentrations (1%, 2%, 3%, 4% and 5%). Out of all these concentrations, the strains under 5% were considered as highest production of PHB. On comparing with the PHB production at 4%, showed the slight increased production rate was observed at 5%. The sugar content of Groundnut oil cake was standardized with standard DNSA method. IR (FTIR) spectra revealed the presence of PHB functional groups, showed 3 major and 3 minor peaks were observed at 235nm.

KEY WORDS: **Poly- β-hydroxybutyrate (PHB), Production, Optimization, cheap substrate - Groundnut oil cake, FTIR spectra.**

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INTRODUCTION

Synthetic Polymers obtained from petrol causes air pollution only because they are not degradable in soil for a long time. For this reason, a microbial plastic poly-β-hydroxybutyrate (PHB) has gained importance because of its easily degradable nature. Environmental pressures are forcing on polymer manufactures to consider biodegradable polymers as an alternative polymeric material. PHB and poly- β- hydroxyalkanoic acids, biodegradable thermoplastics can be produced from a wide range of substrates by using bacteria [1]. To combat challenges due to environmental pollution, Poly-β-hydroxy butyrate (PHB) has been found as eco-friendly and best alternative biopolymers [14]. PHB is a biodegradable and biocompatible thermoplastic produced by various microorganisms [2]. PHB is a partially crystalline thermoplastic & possesses material properties similar to those of polypropylene [5]. The substrate, Ground nut oil cake was used as a source of carbon and therefore it has been purposefully chosen for optimization of PHB producing *Bacillus sp.*

MATERIALS AND METHODS

QUALITATIVE SCREENING FOR THE PRODUCTION OF PHB USING SUDAN BLACK STAINING TECHNIQUE [17].

The isolated bacterial strains were screened for PHB production. As a preliminary step, screening of PHB producers were carried out using viable colony staining technique depending on sudan black absorption pattern. The cultures were grown on minimal media supplemented with glucose (2%) as a sole carbon source, incubated at 40ºC for 48hrs. After incubation, the plates were flooded with Sudan black B solution for the detection of microbial intracellular lipid granules and kept undisturbed for 20 mins. The excess of Sudan black solution was drained off. Viable colony staining technique was selected in order to reveal the different pattern of Sudan black absorption seen on the agar plate.

Preparation of Sudan Black B Solution [10]

The Sudan Black B solution was prepared by dissolving 0.3 gm of Sudan black B powder in 75 ml of 95% ethanol.

PHENOTYPIC CHARACTERIZATION

All the isolates were subjected for partial identification based on various biochemical tests as per Bergy's manual of systematic bacteriology [8].

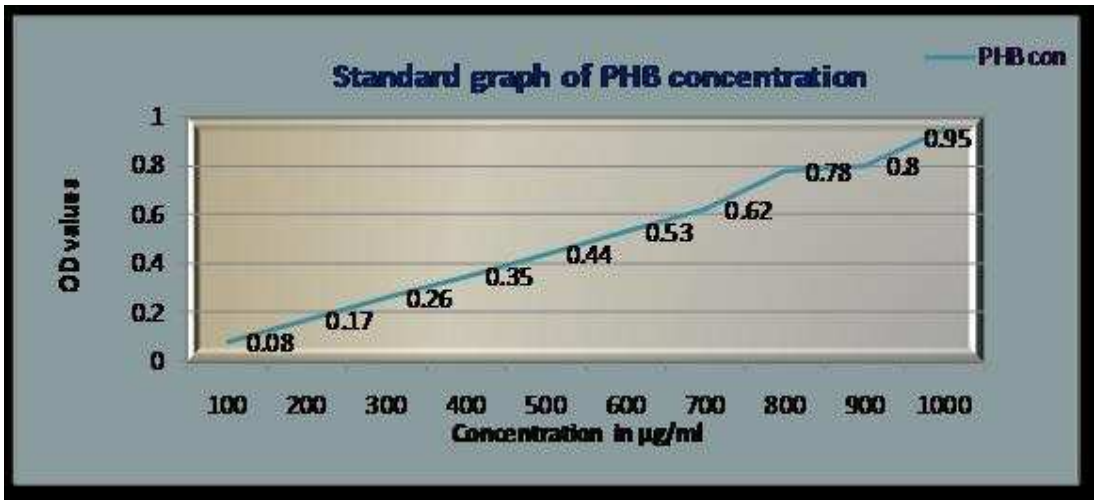
EXTRACTION OF PHB BY CHLOROFORM EXTRACTION METHOD

10ml of the bacterial cultures (24-96 hrs) grown in minimal broth was retrieved at an interval of 24 hrs and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was suspended with 2.5ml of 4% sodium hypochlorite solution and 2.5ml of chloroform. The pellet suspension was incubated at 30ºC for 1 hr. After incubation, the suspension was centrifuged at 1,500 rpm for 10 min. After centrifugation, three phases were obtained. The upper phase of hypochlorite solution which was removed and the middle phase (chloroform containing undisturbed cells) was separated by filtration from the bottom phase (chloroform with PHB) [15].

SPECTROPHOTOMETRIC ANALYSIS (LAW AND SLEPECKY METHOD)

Extracted crude PHB sample (5-50 µg) was digested by heating in concentrated H_2SO_4 at 100˚C for 10 min [13], estimated at 235 nm in U-V visible spectrophotometer with internal standard of crotonic acid in the range of 10-100 µg/ml. The digested sample of crude PHB was scanned in the range of 190-700 nm against concentrated H_2SO_4 as blank in U-V visible spectrophotometer.

FIGURE 1
Standard Graph of PHB Concentration



OPTIMIZATION OF PH AND TEMPERATURE FOR PHB PRODUCTION

Minimal broth was prepared (Glucose-10gm, Dipotassium hydrogen phosphate-7gm, Diammonium sulphate-1gm, Magnesium sulphate-0.5gm, Distilled water-1000ml), sterilized and inoculated with *Bacillus* sp. The temperature and pH was adjusted as (5, 7 and 9) and incubated at 30ºC, 37˚C and 40˚C respectively followed by extraction of PHB by spectroscopic analysis (Systronics, UV-VIS spectrophotometer 108).

OPTIMIZATION OF CHEAP SUBSTRATE (GROUNDNUT OIL CAKE) ON PHB PRODUCTION

To the production medium (minimal broth) the cheap carbon source (Groundnut oil cake) was added at different concentration (1%, 2%, 3%, 4% and 5%). The isolates were inoculated and incubated at 37˚C at pH 7.

Processing of Ground Nut Oil Cake

Groundnut oil cake was gelatinized at 100˚C for 15mins and liquefaction were done with α-amylase at 85˚C with pH 5 for 30mins. The hydrolysates obtained were filtered through a muslin cloth and the clear hydrolysate containing reducing sugar was used as the sole carbon source. The reducing sugar in the hydrolysate was measured using standard (DNSA) method [9].

FTIR Analysis

In order to know the functional groups present in PHB extract, about 1mg extracted sample of PHB were dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film and was subjected to FTIR analysis by using FTIR spectrophotometer. Spectra were recorded in 4000 cm^{-1} to 400 cm^{-1} range.

RESULTS

Phenotypic Characterization

All the isolates were subjected for partial identification based on various biochemical tests according to Bergy's manual of systematic bacteriology. All the isolates were confirmed as *Bacillus* sp. (Table 1).

TABLE 1
Biochemical Tests results for the Isolates

Tests	Strain B6	Strain B7	Strain B8	Strain B10	Strain B11
Gram staining	+	+	+	+	+
Indole	-	-	-	-	-
Methyl red	-	-	-	-	-
VP	+	-	+	-	+
Citrate	+	+	+	+	+
Glucose	A	A	A	A	A
Lactose	-	-	-	-	-
Sucrose	A	A	A	A	A
Mannitol	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Urease	-	-	-	-	-
TSI	A/A	A/A	A/A	A/A	A/A
Nitrate reduction	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+

A – Acid production

Screening of PHB Producers Using Sudan Black Staining Technique

Initially few isolates were screened using Sudan black technique. Blue black granules were observed which is shown in plate 1 and the intensity of the color increases as the increased amount of PHB content more in the colonies. Among the 5 isolates (B6, B7, B8, B10 and B11) the strain B8 reveals the abundance of blue color granules [11].

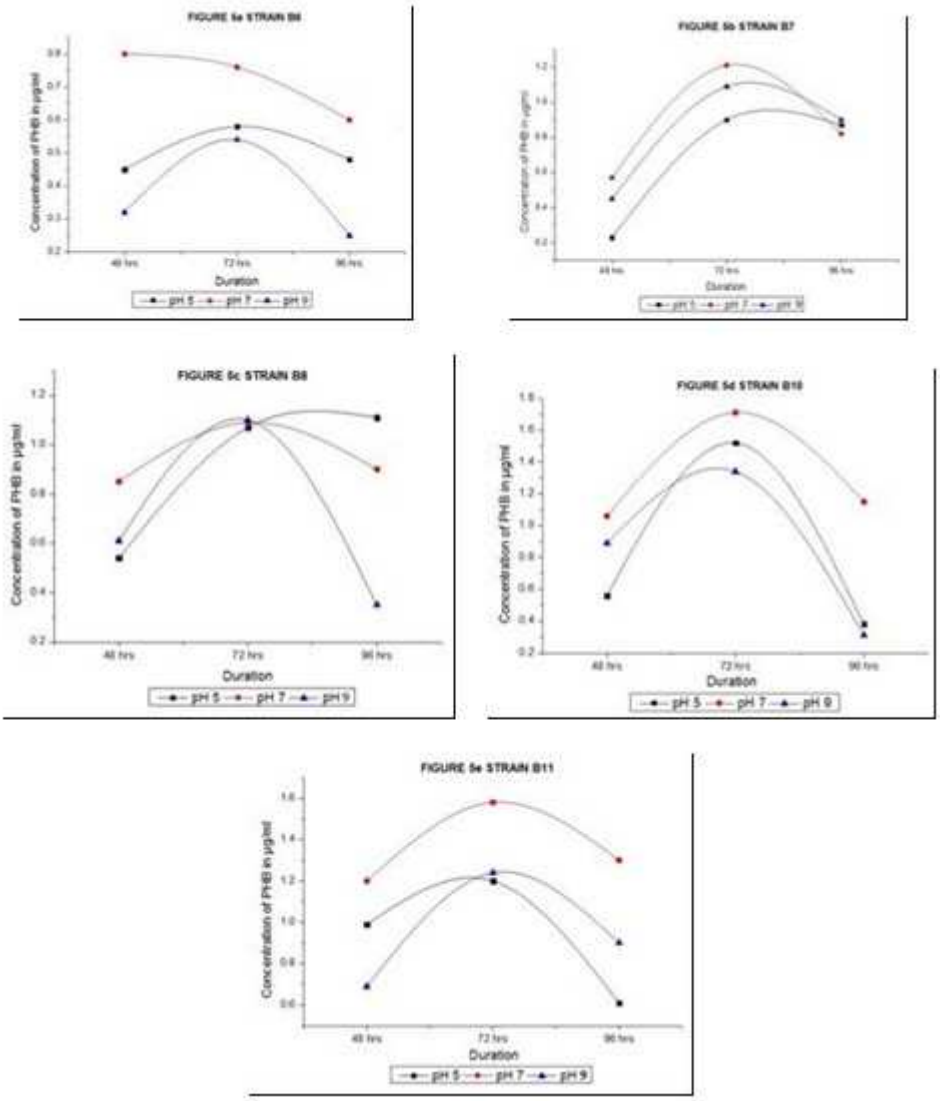
Recovery of PHB

Recovery of PHB was done by extraction method (Chloroform extraction method).

Optimization of pH for the Production of PHB

The selected strains B6, B7, B8, B10 and B11 were optimized for the maximum production of PHB under physical parameters such as pH and temperature. Optimization of the production of PHB under various pH (5, 7 and 9) were analyzed at standard temperature (37°C). The maximum production of PHB was observed at pH 7 for the strains B6, B7, B8 and B11. The production of PHB were compared under various duration (48, 72 and 96 hrs) showed, the maximum production rate was observed at 72 hrs for all the strains. The strains B11 (1.58µg/ml) and B10 (1.71µg/ml) showed the maximum production of PHB at 72 hrs, when compared with other strains. The production of PHB was observed during the period of 48hrs to 72hrs and the reduction in the production of PHB was observed after 72hrs (FIGURE 5).

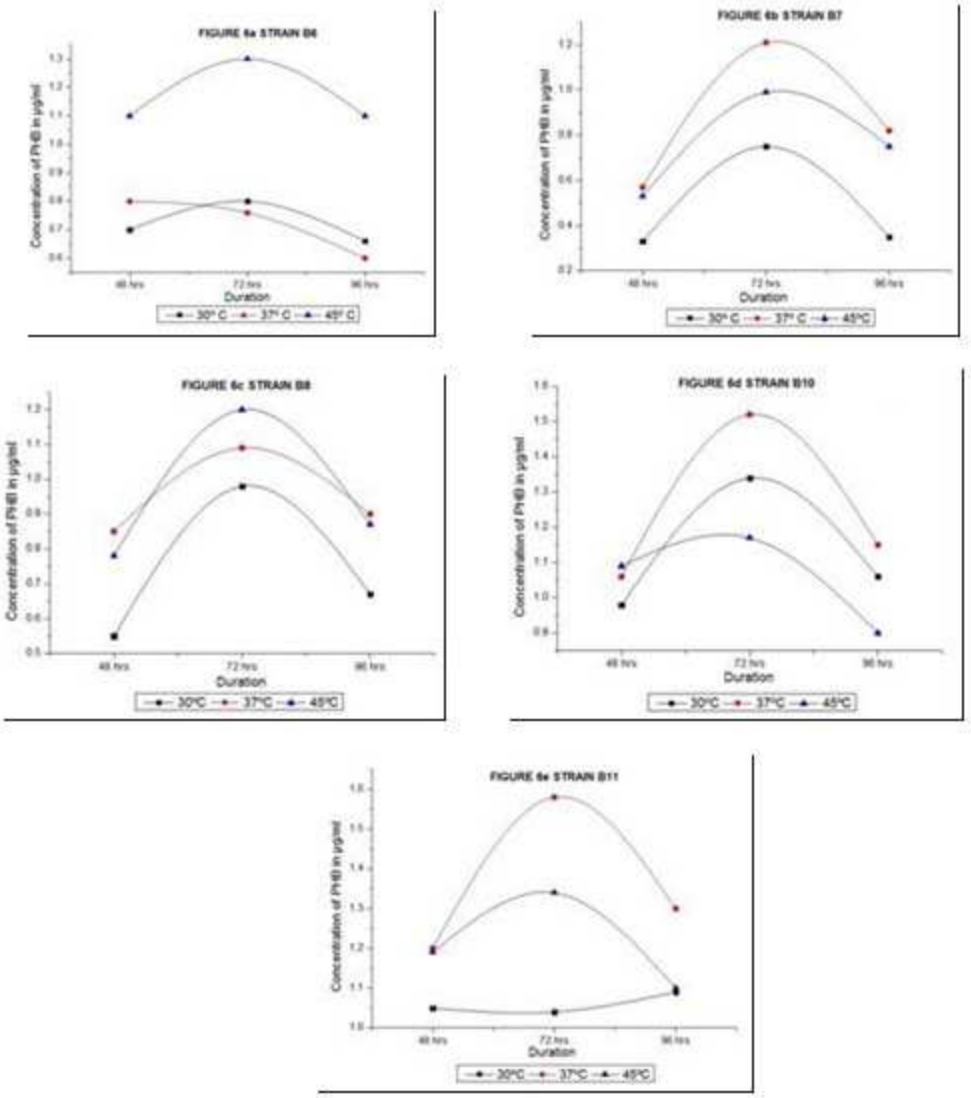
FIGURE 5
Optimization of pH for the Production of PHB at Standard Temperature 37ºC



Optimization of Temperature for the Production of PHB

Optimization of PHB production under various temperatures (30ºC, 37ºC and 45ºC) was analyzed at standard pH 7. The maximum production of PHB was observed at 37ºC. The strain B6 (1.3µg/ml) and B8 (1.2 µg/ml) showed the maximum production of PHB at 45ºC and the moderate production was observed at 37ºC at 72hrs (0.76µg/ml). The strain B7 (1.21 µg/ml), B10 (1.52 µg/ml) and B11 (1.58 µg/ml) showed the maximum production of PHB at 72 hrs at 37ºC (FIGURE 6).

FIGURE 6
Optimization of Temperature for the Production of PHB at Standard pH 7



Optimization of Cheap Substrate (Groundnut Oil Cake)

The cheap carbon source (Groundnut oil cake) was used at different concentration (1%, 2%, 3%, 4% and 5%). The maximum PHB production was observed at 5% utilization of groundnut oil cake as a substrate. Out of all isolates, the strains B8 (0.164µg/ml) and B10 (0.162µg/ml) showed the highest PHB production at 5% utilization at 72 hrs of PHB production as shown in (FIGURE 2 and 3).

FIGURE 2
Effect of Substrate Concentration on Production of PHB

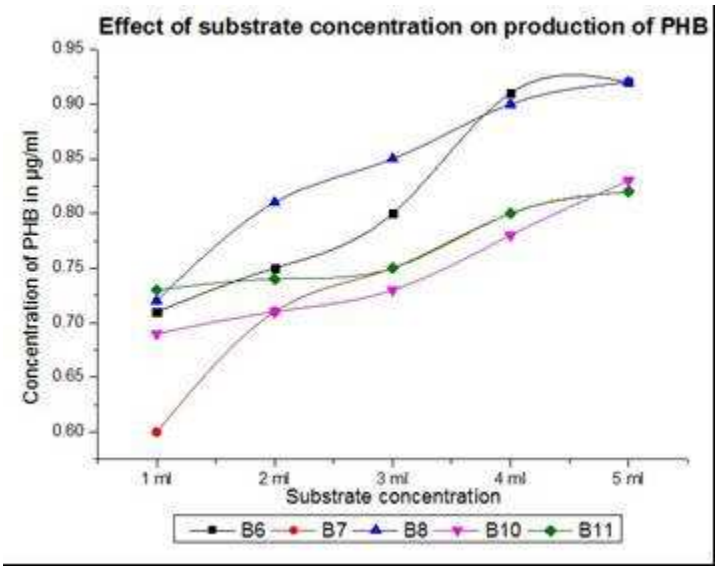
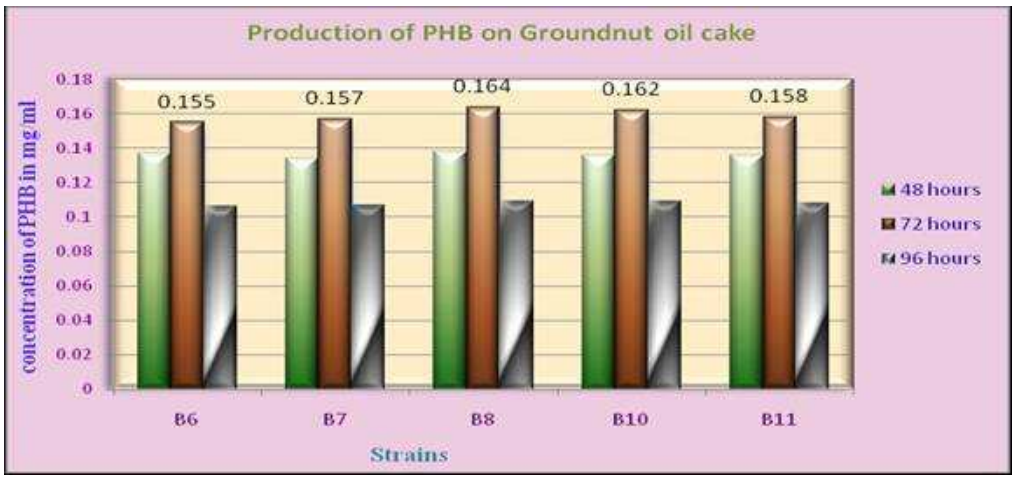


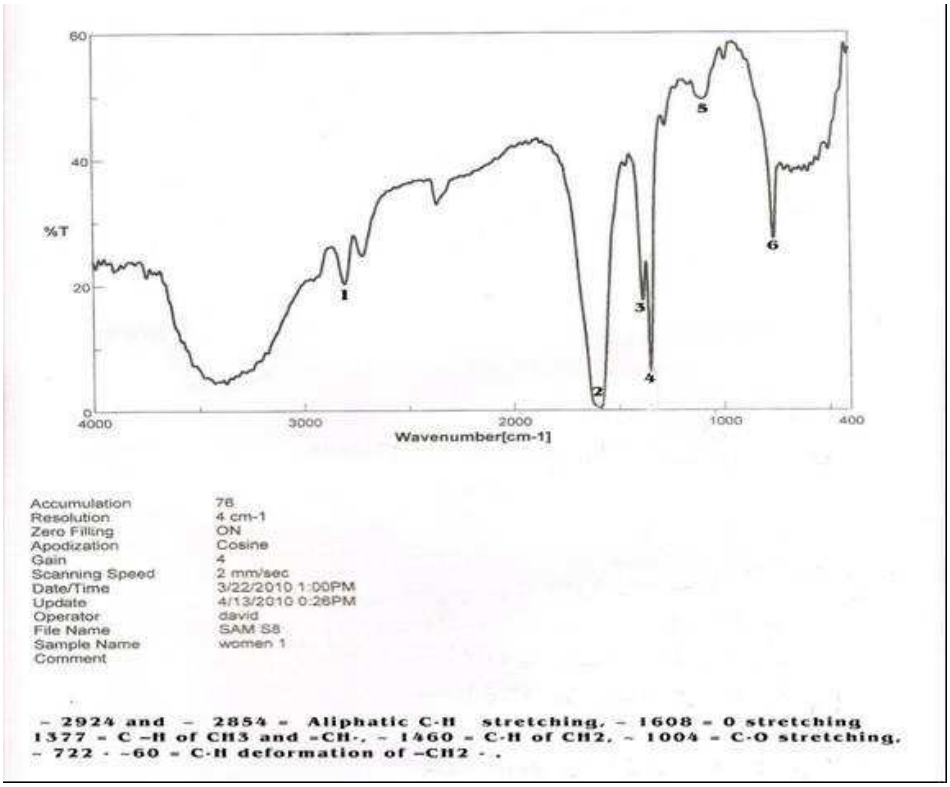
FIGURE: 3
Production of PHB On 5% of Ground Nut Oil Cake



FTIR (FOURIER TRANSMITTANCE INFRARED ANALYSIS)

The spectrum in fig.4 showed the results of the cumulative absorbance for all chemical species present in the sample. These spectra dominated by absorbance for major cellular constituent i.e., PHB. IR spectrum of the extracted product showed PHB content. IR absorption peaks of the product ranges from 2924-522cm^{-1}. (FIGURE 4).

FIGURE 4
FTIR ANALYSIS



DISCUSSION

Out of 5 *Bacillus* sp., (B6, B7, B8, B10 and B11) the strains B6, B8, and B11 showed the maximum production of PHB at 37°C for pH 7. Using the cheap substrate Groundnut oil cake, the strains B8 and B10 showed the highest PHB production at 5% concentration. The PHB synthesized by the cells under growth limiting factor [12] , Since PHB accumulation as an intracellular process, its extraction needs the cell lysis, PHB accumulating cells are known to become fragile and therefore easily lysed. PHB containing cells of *Bacillus* sp., were lysed by sodium hypochlorite solution [16].

Bacillus sp produced the maximum PHB production with the time dependent manner, and observed to be the highest yield 5.311g/L was obtained at 72 h of growth. The stationary condition of growth was attained after 72 hrs. The optimum bacterial growth leads to optimum PHB accumulation which was depends upon the composition of the media under optimized conditions [17]. Effect of pH in the medium showed a strong influence on the production of PHB. The maximum production rate for all the strains were observed at pH 7. The next higher level of production was observed at pH 9 and minimum production was observed at pH 5. The influence of pH on the growth of *B. cereus* revealed that most of the strains tested had their optimum growth at pH 9. However, pH 1 and 12 were found to be inhibitory to growth of *B. cereus*. The finding and the agreement with the work of [4] who reported that pH 4.9 - 9.3 permitted growth of *B. cereus* in laboratory media. The optimum PHB production was obtained at pH range from 6.0 to7.5 [4]. The similar results were observed from [17], the majority of *Bacillus megaterium* showed the mesophilic character with temperature optima between 30°C and 45°C, without any added

growth factors. Indeed, the ability of *Bacillus megaterium* to accumulate PHB was so dominant that the PHB contents in the cells could reach up to 32% of the cell dry weight. PHB provides a reserve of carbon and energy, accumulated as intracellular granules which can be extracted from wide range of bacteria [16].

The cheap carbon source (Groundnut oil cake) was used at different concentration (1%, 2%, 3%, 4% and 5%). The maximum PHB production was observed at 5% utilization of groundnut oil cake as a substrate. Out of all isolates, the strains B8 (0.164µg/ml) and B10 (0.162µg/ml) showed the highest production at 5% utilization at 72hr of PHB production. FTIR spectra predicted the presence of functional groups of PHB [15] i.e. aliphatic C-H, = O stretching, = C-H deformation, = C-H, = CH, = C-O. PHB and copolymers are known to contain these functional groups [10]. For more clarification of chemical structure of extracted PHB and further studies are needed. The absorption band obtained from 2924 cm^{-1} to 2854 cm^{-1} streching as aliphatic C-H band (peak one), The absorption band obtained at 1608 cm^{-1} as = O streching (Peak two), The absorption band obtained at 1460 cm^{-1} as C-H of CH_3 streching (Peak three). The absorption band obtained at 1377 cm^{-1} as C-H of CH_2 streching (Peak four). The absorption band obtained at 1004 cm^{-1} as C-O streching (peak five). The absorption band obtained at 722 cm^{-1} as = C-H deformation of - CH_2 streching (peak six).

CONCLUSION

The use of cheap substrate to produce PHBs has gained much attention in recent years, because the selected strains can use groundnut oil cake as a alternate carbon sources, resulting in a decrease in the cost of production of PHBs. Effective six PHB producing strains were selected for the maximum production of PHB under standard temperature and pH, the results showed that the strains B6, B8 and B11 to be the maximum producer at 37°C for pH 7. The maximum PHB production was observed at 5% utilization of groundnut oil cake as a substrate for all the strains. Six peaks observed from FTIR spectra revealed the functional groups of PHB. From the above analysis it was concluded that the use groundnut oil cake as an alternate carbon sources for PHB production.

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