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INFLUENCE OF NUTRITIONAL AND ENVIRONMENTAL PARAMETERS ON PHB PRODUCTION BY BACTERIA ISOLATED FROM CONTAMINATED SOILS

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ABSTRACT

Polyhydroxybutyrates (PHBs) are an attractive alternative to conventional non-biodegradable plastics, as these have physical properties similar to polypropylene and are also fully biodegradable. In the present study, an attempt was made to isolate efficient PHB producing bacteria from effluent discharge sites of different industries. Using Sudan Black screening method, 20 PHB positive bacteria (PHB yield varying between 12.11 to 82.53 mg/ml) were isolated from contaminated soil samples. Chemical nature of the extracted polymer was confirmed using FTIR analysis. Based upon their biochemical characterization, the isolates were tentatively found to belong to three prominent genera, *viz*., *Bacillus*, *Staphylococcus* and *Acinetobacter*. Culture medium conditions having glucose as carbon source, ammonium sulphate as nitrogen source, C:N ratio as 20:1, pH at 7.0, and an incubation temperature of 30^0C were found to support maximum PHB production by all the isolates. At optimized conditions, a few isolates (IBB, ITF and ITG) exhibited significant PHB yields, thus showing a potential for their possible industrial exploitation.

KEY-WORDS: Biodegradable, biochemical characterization, culture medium parameters, optimization, PHB.

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INTRODUCTION

Synthetic plastics provide a range of utilities in the civilization of mankind, but at the same time, the accumulation of these non-degradable plastics in the environment is a menacing drawback which is increasing day by day. In the face of these ecological facts, production of biodegradable polymers from renewable resources has become the need of the hour. 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. Amongst the various existing biopolymers, polyhydroxyalkonates (PHA) are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a large extent. Polyhydroxyalkanoate is a biodegradable microbial polymer which is accumulated in bacteria as an intracellular storage granule in the presence of excess carbon source and limited nitrogen source[1]. Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments[2], unlike many of the "so-called" biodegradable plastics made synthetically. PHAs are composed mainly of poly-beta hydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV), although other forms are possible. Many different types of PHAs exist, but, only two forms *i.e*., PHB homopolymer and 3HB-3HV (-3-hydroxybutyrate-3-hydroxyvalerate) copolymer are commercially produced. It has been reported that the accumulation of PHB by microorganisms can be stimulated under unbalanced growth conditions, *i.e*., when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C:N ratio of the feed substrate is higher[3-6]. It is synthesized as an intracellular storage material accumulating as distinct white granules, which are clearly visible in the cytoplasm of the cell. During adverse conditions, these PHB storage granules are used by the cell as an internal reserve of carbon and energy. Many bacteria including those in the soil are capable of PHB production and breakdown[7]. PHB differentiates itself from other biodegradable plastics as it has unique properties like being insoluble in water, highly resistant to hydrolytic degradation, oxygen permeability, UV resistant; whereas other biodegradable plastics are moisture-sensitive and water-soluble. PHB has poor resistance to acids and bases, is soluble in chloroform and other chlorinated hydrocarbons and is biocompatible; hence it is also suitable for medical applications. Although the biodegradable polyesters display a special interest due to their possible use as substitutes of common plastics, but their production on a large scale is limited because of the relative expense of the substrate and low polymer production[8]. The higher production costs, especially raw material costs, make it difficult for PHB plastics to compete with conventional petroleum-based plastics in the commercial market place. Hence, alternative strategies for PHB production are being investigated. The success of the biodegradable plastic strategy largely depends on the isolation of potent PHB producing bacteria and optimizing culture medium parameters for maximum PHB biosynthesis. So, keeping these points in view, the present study was addressed to isolate PHB producing bacteria from industrial effluent discharge sites (such contaminated soils are rich in organic matter content, and therefore have the potential for harboring a large population of bacteria, which are likely to be potential PHB-producers) of three different industries, and to optimize growth and culture conditions such as incubation temperature and pH; and medium constituents like carbon and nitrogen sources for maximizing PHB production by them.

MATERIALS AND METHODS

Standard bacterial culture

A standard PHB positive bacterial strain, *viz*., *Cupriavidus necator* - MTCC 1472 (submitted as *Alcaligenes eutropha*), was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. This was used as the

reference strain for comparison of the native isolates collected in the present study.

Soil Sample Collection

For isolation of PHB producing bacteria, soil samples were collected from effluent discharge sites of three different industries, *viz.,* sugarcane mill, thermal power plant and a brewery; and were stored in plastic bags at low (4^0C) temperature. For further processing, the collected samples were air dried at room temperature, and then crushed with the help of pestle & mortar for further analysis.

Bacterial isolation from collected soil samples

The soil samples collected from effluent discharge sites of different industries were subjected to serial dilution plating. For this, 10 gram of each soil sample wassuspended in 90 ml of sterile distilled water, shaken vigorously and serially diluted in sterile distilled water. Dilutions ranging from 10^{-5} to 10^{-8} were plated on standard nutrient agar medium. After 48 hrs of incubation at 30^0C, the total number of bacterial colony forming units (cfu) of each soil sample was enumerated. The well-formed colonies were then studied for their morphological characteristics on the basis of their physical appearance (colour, size, shape and texture), and those showing remarkable differences in their morphology were selected and re-streaked on nutrient agar plates to obtain pure cultures.

Screening of isolates for PHB production using Sudan Black dye

All the representative pure isolates were screened for PHB production using the lipophilic stain Sudan Black B on agar plates, and under light microscope[9].

Screening for PHB on solid agar

Individual bacterial isolates were streaked on nutrient agar plates (4-5 isolates on one plate), and the plates were incubated at 30^0C for 24 hrs. Ethanolic solution of 0.3% (w/v in 70% ethanol) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. The plates were then destained by washing with ethanol (96%) to remove excess stain from the colonies. The colonies that retained their black colour after destaining were attributed as PHB producing strains[10].

Screening for PHB production under light microscope

For microscopic studies, smears of different colonies were prepared on glass slides, heat fixed and stained with a 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. The colonies were decolorized by immersing the slides in xylene, and were then counterstained with safranin (5% w/v in sterile distilled water) for 10 sec. Bacterial cells appearing black under the microscope were considered PHB producing strains while others were marked as negative[11]. All the positive isolates were assigned code numbers based on their source of isolation.

PHB extraction and quantification

Polyhydroxybutyrate polymer was extracted and the amount of PHB produced was calculated from the standard curve prepared by using commercial poly-β-hydroxybutyrate (Sigma-Aldrich) as per the method detailed by Law and Slepecky (1961)[12]. The PHB positive bacterial culture growth was pelleted at 10,000 rpm at 4^0C for 10 min. The pellet was then washed with acetone and ethanol to remove unwanted materials, resuspended in equal volume of 4% sodium hypochlorite and incubated at room temperature for 30 min. The mixture was then centrifuged at 10,000 rpm for 10 min. to sediment the lipid granules. The supernatant was discarded, and the pellet was washed successively with acetone and ethanol. The pelleted polymer granules were dissolved in hot chloroform and filtered through Whatman No. 1 filter paper (previously treated with hot chloroform). To the filtrate, 10 ml of hot concentrated H_2SO_4 was added, which converts the polymer to crotonic acid, turning it into a brown colored solution. The solution was cooled and absorbance was read at 235nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer[13]. The quantity of PHB produced was determined by referring to the standard curve. Based on the quantification high PHB producing isolates were selected for further FTIR studies.

Preparation of standard curve

Pure PHB (Sigma, USA) was used to prepare the standard curve of PHB. Two gram of PHB was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min to convert PHB into crotonic acid, which gave 200 mg/ml of crotonic acid. From the above stock, working solutions were prepared by diluting it to obtain different concentrations ranging between 10 mg/ml to 150 mg/ml. Absorbance of all the dilutions was read at 235nm against a concentrated H_2SO_4 blank on UV-VIS spectrophotometer, and the standard graph was made by plotting the various concentrations on the x-axis and the respective optical densities on the y-axis. The standard curve was used for estimation of PHB yield of the bacterial isolates.

FTIR analysis of PHB extracts

The extracted polymer from selected PHB positive isolates was analyzed qualitatively using FTIR spectrophotometer (Bruker alpha, Platinum ATR- Attenuated total reflectance). An aliquot of approximately 1mg of the polymer extracted from two highly efficient PHB producers, from the standard strain MTCC 1472 (*Cupriavidus necator*), and the standard PHB (Sigma-Aldrich) was subjected to FTIR analysis. Spectra were recorded at 4000 cm^{-1} to 375 cm^{-1} range using attenuated total reflectance. 24 scans were averaged to get the IR spectra and the spectra were recorded with $4cm^{-1}$ resolution. The FTIR spectra of the two bacterial isolates were compared with that of the standard PHB (Sigma) and from the standard strain MTCC 1472.

Morphological and biochemical characterization of PHB positive isolates

The PHB positive isolates were grown on nutrient agar plates and their colony morphology was recorded. The morphological characteristics of the representative bacterial isolates (from each soil sample) showing differences in their physical appearances were recorded under four major headings, *viz*., size, colour, texture and shape. All these isolates were also studied under the microscope with respect to their cellular morphology and Gram staining properties[14]. Biochemical characteristics of the isolates were studied following the standard microbiological methods[15]. Identification of isolates was carried out on the basis of the results of morphological, cellular and biochemical characters studied. Molecular characterization of the isolates is underway.

Optimization of culture medium parameters for maximum PHB production

Different factors *viz*., carbon and nitrogen source, C/N ratio, pH, and incubation temperature play an important role in PHB production rate. Therefore, the effect of all these parameters on PHB production by the PHB positive isolates was studied by varying all the conditions within a defined range.

Optimization of different carbon sources

The effect of different carbon sources on PHB production was determined by raising the cultures of the PHB positive isolates in 100 ml of minimal salt medium (MSM)[16] supplemented with different carbon sources such as glucose, fructose, sucrose, maltose and arabinose at 2% concentration. Cultures were incubated at 30^0C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB produced by the isolates was quantified spectrophotometerically (as described earlier), and based on the yield, the best carbon source was determined**.**

Nitrogen source optimization

The PHB positive isolates were inoculated in 100 ml of MSM broth containing the best carbon source and different nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate and yeast extract) at 1% concentration. After 48 h of incubation at 30^0C, PHB yield was determined for all the isolates, and the best nitrogen source was selected on the basis of their yield. Further, the effect of different concentrations of the best nitrogen source on PHB production was also studied by determining the PHB yield upon growing the isolates in MSM supplemented with the best C-source and different concentrations (0.5, 1.0 and 1.5 g/l) of the best N-source.

Optimization of Carbon to Nitrogen Ratio (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 was also determined. For this, cultures were inoculated in MSM supplemented with different ratios of concentrations of the best C and N source (C/N ratio as 10:1, 15:1, 20:1 and 25:1). Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 h. After incubation, PHB yield was quantified spectrophotometerically, and based on the yields the most favourable C/N ratio was determined.

Effect of pH on PHB production

For pH optimization, cultures of the selected isolates were raised in MSM supplemented with the best C and N source having different pH, viz., 6.0, 7.0 and 8.0. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hrs. After incubation, PHB yield was quantified spectrophotometerically, and the pH exhibiting maximum yield was determined.

Effect of temperature on PHB production

The effect of different temperatures on PHB production was determined by inoculating the cultures in MSM supplemented with the best C and N source and then incubating at different temperatures viz., 25^0C, 30^0C, 35^0C, 40^0C, and 45^0C. After 48 h of incubation at respective temperatures, PHB yield was quantified spectrophotometerically; based on the yields the optimum temperature for maximum PHB production was determined.

RESULTS AND DISCUSSION

Isolation and screening of PHB producing bacteria

Soil samples were collected from effluent discharge sites of three different industries in Haryana (Table I) and total bacterial population was enumerated by making serial dilutions of each soil sample and plating appropriate dilutions on nutrient agar medium. Based upon the morphological differences in their colony characteristics (size, shape, colour and texture), a total of 31 representative bacterial colonies (11 from the effluent discharge site of sugarcane industry; 13 from thermal power plant effluent site; and seven from brewery discharge site) were picked, purified and maintained as pure cultures for further screening using Sudan Black dye. The screening was done by staining the isolates with Sudan Black B on petri plates as well as under the microscope. Sudan Black dye has been used as a screening measure for PHB production by several workers[17-20]. It was observed that out of 31 isolates, as many as 20 isolates (08 each from effluent discharge sites of sugarcane and thermal industry, and four from the brewery discharge site) accumulated PHB; appearing as blue/ black droplets in the cells under the microscope (Fig. Ib), and as blue/ black colonies upon staining on plates with Sudan Black dye (Fig. Ia). All the positive isolates were assigned code numbers (ISA to ISH, ITA to ITH and IBA to IBD) based on their source of isolation (Table I).

PHB Extraction and Quantification

All the 20 Sudan Black B positive isolates and the standard strain *Cupriavidus necator* (MTCC 1472) were subjected to quantitative estimation of PHB[12]. By referring to the standard curve, the PHB yield was calculated, and it was found to vary between 12.11mg/ml (ISA isolate) to 82.53mg/ml (IBB isolate) (Table II). Highest PHB producers (with an average of 50.42 mg/ml PHB production) were obtained from the brewery effluent site soil samples, followed by isolates from the thermal industry effluent discharge site (with 42.81 mg/ml as average PHB production) and isolates with lowest PHB yield belonged to the sugarcane industrial effluent site samples (an average of 24.31 mg/ml of PHB). However, the PHB yield of the reference strain MTCC 1472 was found to be higher (144.23 mg/ml) than the isolates. The 20 PHB positive isolates were further subjected to biochemical characterization, and optimization of medium constituents to maximize PHB production.

FTIR Analysis for functional group identification

FTIR spectra of the extracted polymer from two high yielding isolates (IBB and ITG) were recorded in the range of 4000 cm^{-1} to 375 cm^{-1}. The various spectral peaks signify specific rotations around carbon atoms specific to certain functional groups (Fig. IIc, IId). The peaks obtained at 1732 cm^{-1} (for IBB) and 1731 cm^{-1} (for ITG) represent the C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structures such as PHB[21]. Another marked peak for the ester carbonyl bond was observed at 1645 cm^{-1}. Other absorption bands at 1455 cm^{-1} and 2923/2921 cm^{-1} corresponding to $–CH_2$ and –CH groups were also obtained for the two isolates. These peaks correspond to the peaks obtained for PHB extracted from the standard strain *Cupriavidus necator* MTCC 1472 at 1728 cm^{-1}, 1636 cm^{-1} and 2958 cm^{-1} (Fig. IIb), and also to the peaks obtained for the standard PHB (from Sigma) at 1720 cm^{-1}, 1686 cm^{-1}, 1453 cm^{-1} and 2933 cm^{-1} (Fig. IIa); thus, confirming that the extracted polymer is PHB. The peaks obtained in this study are in consonance with results of similar previous studies[22-25].

Morphological and biochemical characterization of PHB positive isolates

All the 20 PHB positive isolates were subjected to morphological and biochemical characterization. Colony morphology was studied in terms of their size, shape, colour, texture and staining characteristics. Size varied from very small to large, while shapes swirled mainly between circular, ellipses and irregular. Color varied from white to off-white to slightly yellowish. All types of colony textures were obtained such as raised, flat, convex, wrinkled, grainy, slimy etc. Gram staining revealed that a major section of the isolates were Gram positive in nature, there being only one Gram negative isolate among the total 20 PHB positive isolates. Both coccus and bacillus forms were observed and cells could be seen in individual cell forms as well as in diplo- and chain forms. All the PHB positive isolates were subjected to standard biochemical tests and identification was carried out as per the details given in Bergey's Manual of Systematic Bacteriology[15]. On a preliminary basis, the isolates have been found to belong to three genera, *viz.*, *Bacillus, Staphylococcus and Acinetobacter sp.*, (Table III). Molecular identification of the isolates is underway.

Optimization of culture medium constituents and growth conditions for maximum PHB production

The accumulation of PHB by different bacteria has been observed to vary with the type and amount of carbon (C) and nitrogen (N) sources incorporated in the medium, and also with other physical factors such as the pH and temperature of the medium used for growing the bacterial isolates[18]. Therefore, all these factors affecting PHB production by promising bacterial isolates were optimized for maximization of PHB production.

Effect of different carbon sources on PHB production

All the PHB positive isolates were grown in the presence of five different carbon sources (glucose, fructose, sucrose, maltose and arabinose) and the PHB produced by them was quantified for selection of the source showing highest PHB production. The effect of these different carbon sources on PHB yield is shown in Fig. III. The results showed significant differences for the isolates, carbon sources and their interactions as well. Amongst the different PHB isolates, IBB was found to be significantly superior when compared to all other isolates, followed by the isolates ITF and ITG. Among the different carbon sources tested to evaluate their effects on PHB yield, glucose was found to be the best carbon source. It yielded a mean PHB of 52.19 mg/ml. This was followed by fructose with a mean PHB of 43.82 mg/ml. PHB yield was almost comparable for sucrose and maltose (although lower than that with fructose) and least when the MSM medium was supplemented with arabinose. Different workers have tried using different sugars as C-sources; and have observed high PHB yields upon utilization of sugars such as fructose[26], and maltose[13]. However, according to the results obtained in the present study and also on the basis of a

few previous reports[26], it can be concluded that monosaccharides such as glucose and fructose are readily utilized by bacteria and, hence support growth and subsequently PHB production. On the other hand, complex molecules like starch and lactose are not easily utilized for effective PHB production. As the complexity of the carbon sources increases, PHB yield was found to decrease. Similar conclusions have been made in earlier studies[27] also. The interaction effects of the isolate and carbon sources were also found to be significant. The isolate IBB on glucose as the carbon source (2%) recorded the highest PHB yield of 91.24 mg/ml; this was higher than its yield in NA (82.53 mg/ml). The isolate IBB was found to produce significantly higher yield on fructose (75.11mg/ml) as well.

Effect of Different Nitrogen Sources

Fig. IV represents the PHB yield of bacterial isolates in presence of different N sources and the best carbon source (glucose). It was observed that there were significant differences for the isolates, N sources and their interactions. Amongst the isolates, IBB was again found to be a significantly higher PHB producer compared to the other isolates. It produced a mean PHB of 69.32 mg/ml. This was followed by ITF and ITG. Amongst different N sources, ammonium sulpahte was found to be the best N source. It produced a mean PHB of 58.27 mg/ml. The interaction effects of isolates and N sources were also found to be significant. IBB on ammonium sulphate gave the highest PHB yield of 97.53 mg/ml, higher than its yield in nutrient broth. These results are in agreement with the results obtained in earlier reports[26], wherein the highest PHB by *R. eutropha* was observed on MSM medium supplemented with ammonium sulphate. The next promising N sources were ammonium chloride with 45.84 mg/ml and ammonium nitrate with 33.36 mg/ml PHB yields. Yeast extract was found to be the least supporter of PHB production. Several workers have studied the accumulation of PHB by *A. eutrophus* with different salts of ammonium; and similar to the results of the present study, a few have obtained highest PHB yield in ammonium sulphate followed by ammonium chloride [28, 29]. Ammonium sulphate being a simple nitrogen source is probably more readily available than the other complex nitrogen sources. However, contrary to these results, a few studies[13] have reported highest PHB production with ammonium nitrate.

Effect of different concentrations of ammonium sulphate (the best N source) on PHB yield

PHB yields produced by the bacterial isolates when grown on different concentrations of the best N source *i.e.,* ammonium sulphate in presence of the best carbon source (glucose) are furnished in Fig. V. Out of the three concentrations (0.5, 1.0 and 1.5 g/l) of ammonium sulphate tested, it was found that ammonium sulphate at a concentration of 1.0 g/l supported the highest PHB production (with an average of 58.27 mg/ml). In general, there was an increase in PHB production with an increase in ammonium sulphate concentration from 0.5 to 1.0 g/l, but concentrations above 1.0 g/l resulted into a decrease in PHB production by all the isolates. An observed decrease in PHB accumulation upon increasing the ammonium sulphate concentration beyond 1.0 g/l may be attributed to the absence of nitrogen stress condition required for accumulation of PHB. The results of the present study are in accordance with studies conducted by other workers[26, 30,31], who have also reported maximum PHB accumulation at 1.0 g/l. Amongst the different selected isolates, IBB was found to be significantly superior compared to other isolates. At 1.0 g/l concentration of ammonium sulphate, it produced the highest PHB yield of 97.53 mg/ml, and a mean PHB yield of 64.12 mg/ml. In fact, IBB was the highest PHB producer at all levels of ammonium sulphate. ITF and ITG were the next leading PHB accumulators.

***Effect of relative concentration of carbon and nitrogen sources on PHB* production**

Different C:N ratios (10:1, 15:1, 20:1 and 25:1) were maintained using the best carbon and nitrogen sources in the minimal salt medium and their effect on PHB production was studied. The results obtained are presented in

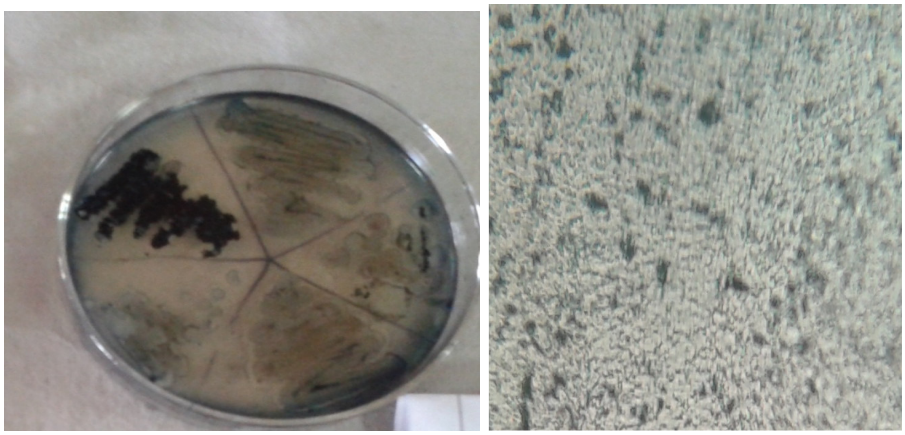
Fig. VI. Amongst the different C:N ratios tested, 20:1 was found to be best C:N ratio supporting the highest PHB production (with an average of 64.45 mg/ml). In all the isolates, there was an increase in PHB production with an increase in C:N ratios upto 20:1 and tapering thereof. This could probably be due to substrate inhibition. Similar observations have been made in previous studies[31, 32]. Out of the various isolates tested, IBB was found to be a significantly higher PHB producer, producing highest PHB yield of 97.99 mg/ml, and a mean yield of 66.30 mg/ml at a C:N ratio of 20:1. ITF and ITG were found to be the next promising isolates.

Effect of pH on PHB production

Out of the different media pH tested, pH 7.0 was found to be optimum for maximum PHB production by all the isolates as shown in Fig. VII. At pH 6.0, all the isolates were found to produce very low yields on pH 6.0, showing that pH 6.0 was not at all suitable for PHB accumulation. Although pH 8.0 was found to support PHB accumulation, but it was lower than that at pH 7.0. pH 7.0 resulted in a mean PHB production of 62.82 mg/ml, while it was 16.02 mg/ml at pH 6.0 and 52.76 mg/ml at pH 8.0. At pH 7.0, the highest PHB of 95.31 mg/ml was produced by IBB which was significantly higher than all other isolates. IBB was again the best isolate with mean PHB production of 89.13 mg/ml. These results are in consonance with some of the previous studies[33] where it has been reported that pH values ranging between 6.0 - 7.5 are optimum for PHB production.

Effect of incubation temperature on PHB production

Effect of varying incubation temperatures on PHB production was studied by maintaining different temperature conditions (25^0C, 30^0C, 35^0C, 40^0C and 45^0C) during incubation of the isolates in a medium prepared using the best carbon and nitrogen sources (Fig. VIII). 30^0C temperature was found to be optimum for maximum PHB production by all the isolates. It yielded a mean PHB of 62.82 mg/ml. This was followed by 35^0C temp with a mean of 60.87 mg/ml. The isolate IBB was found to produce a PHB yield of 95.31 mg/ml and 92.27 mg/ml at incubation temperatures of 30^0C and 35^0C, respectively. Temperatures below 30^0C and beyond 35^0C did not support good PHB yield by any isolate. Similar results have been reported in previous studies[33], wherein it has been concluded that 33^0C incubation temperature is optimum for PHB synthesis under fermentation conditions.



(a) (b)

Figure I
Selected blue black colored colonies after Sudan Black B staining

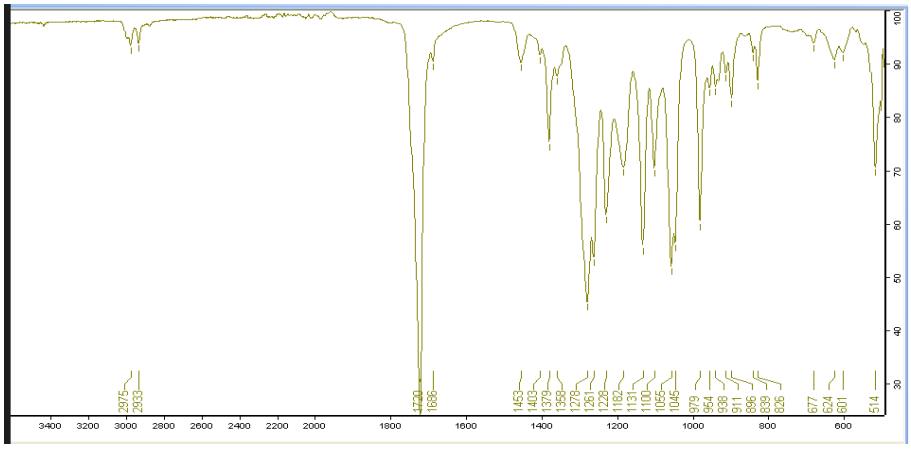


Figure II a
FTIR spectra of standard PHB (Sigma-Aldrich)

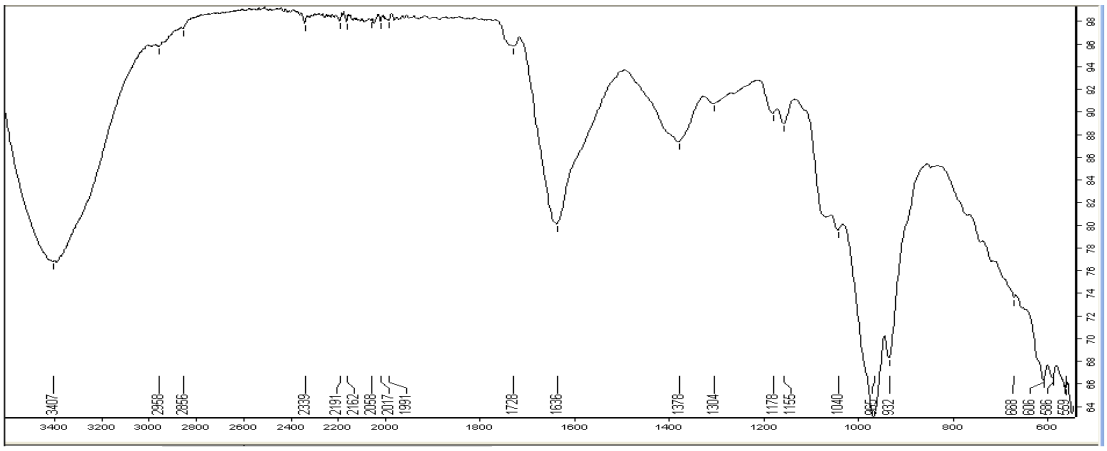


Figure II b
FTIR spectra of PHB extracted from a standard strain Cupriavidus necator (MTCC 1472)

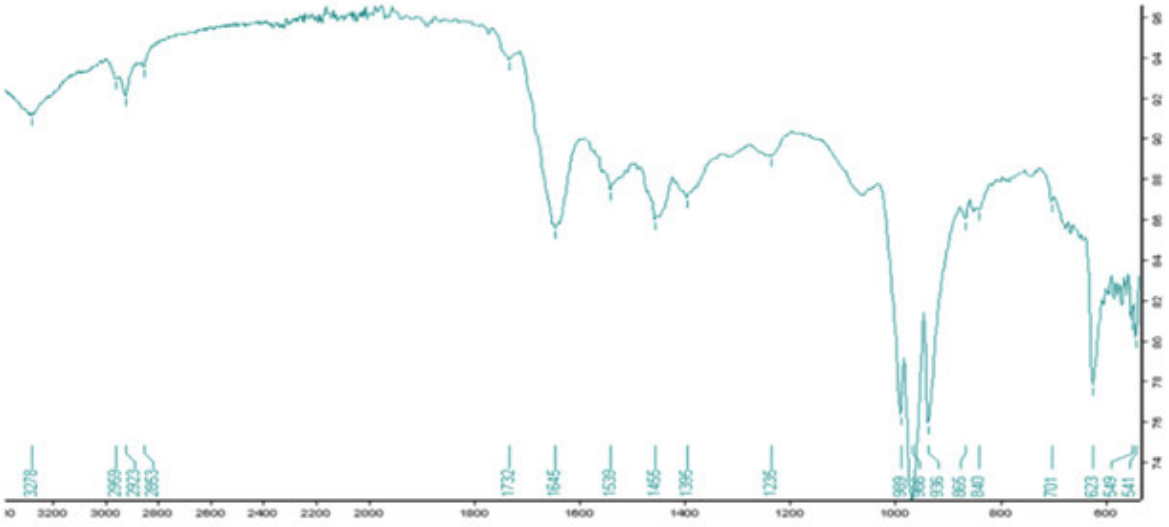


Figure II c
FTIR spectra of PHB extracted from IBB isolate

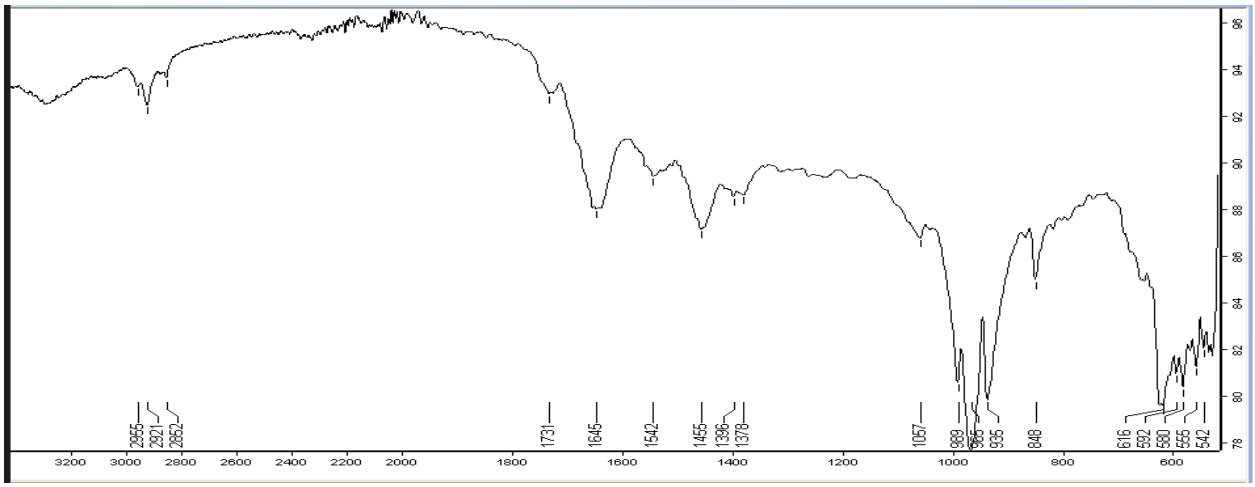


Figure II d

FTIR spectra of PHB extracted from ITG isolate

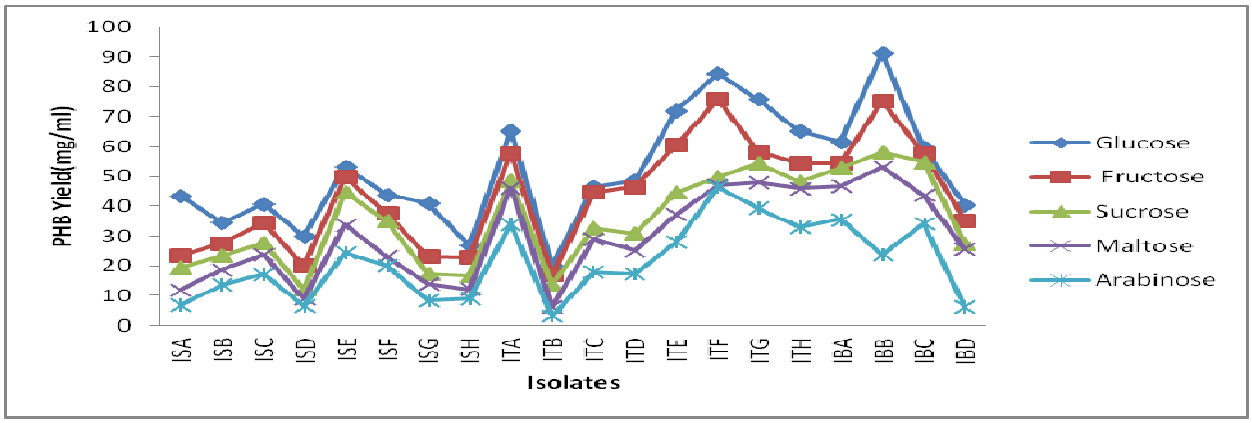


Figure III

PHB production by bacterial isolates as influenced by different carbon sources

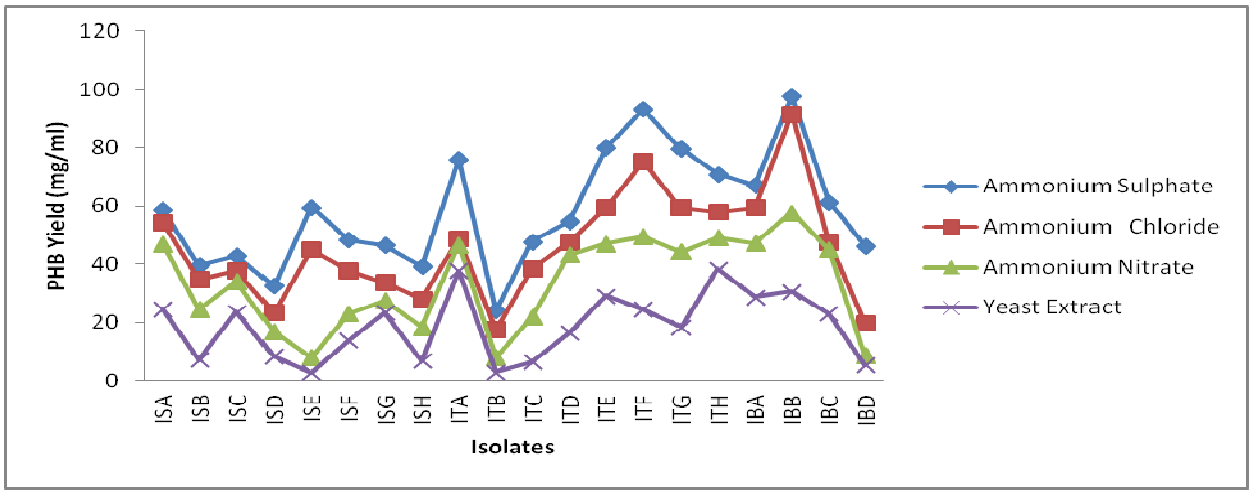


Figure IV

Effect of different N sources on PHB production by the selected bacterial isolate

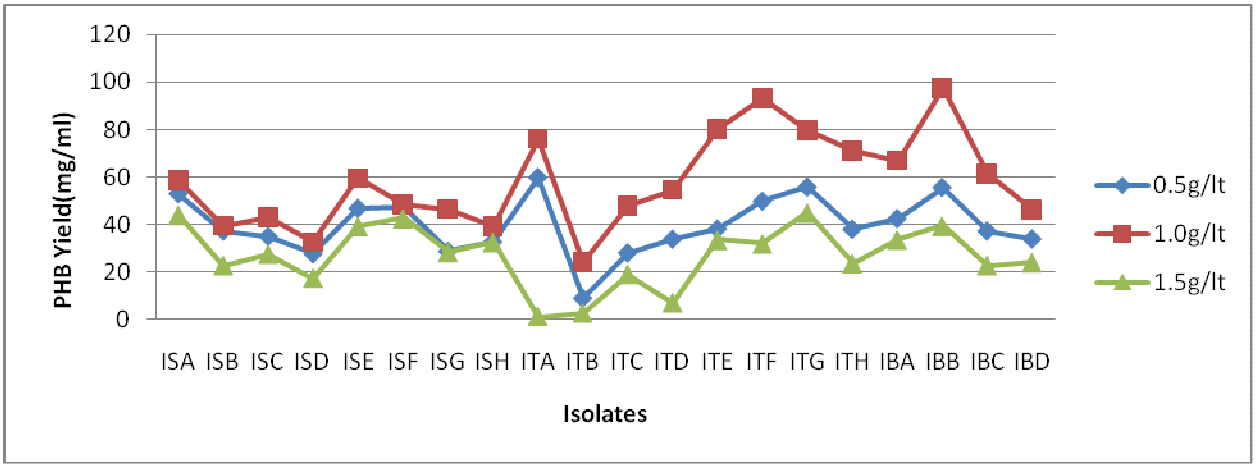


Figure V

Effect of different concentrations of ammonium sulphate on PHB production by the elected bacterial isolates

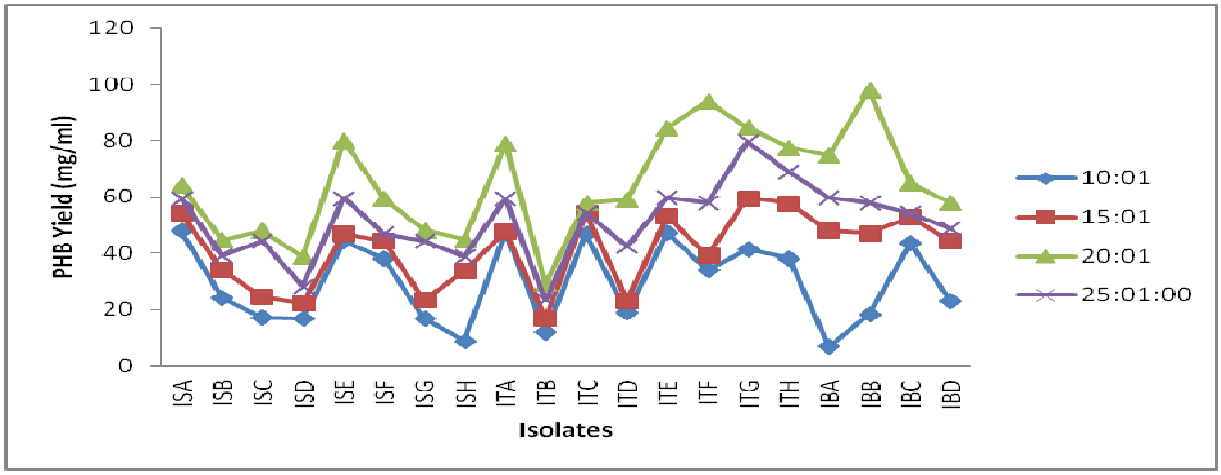


Figure VI

Effect of different C:N ratios of medium on PHB yields by the selected bacterial isolates

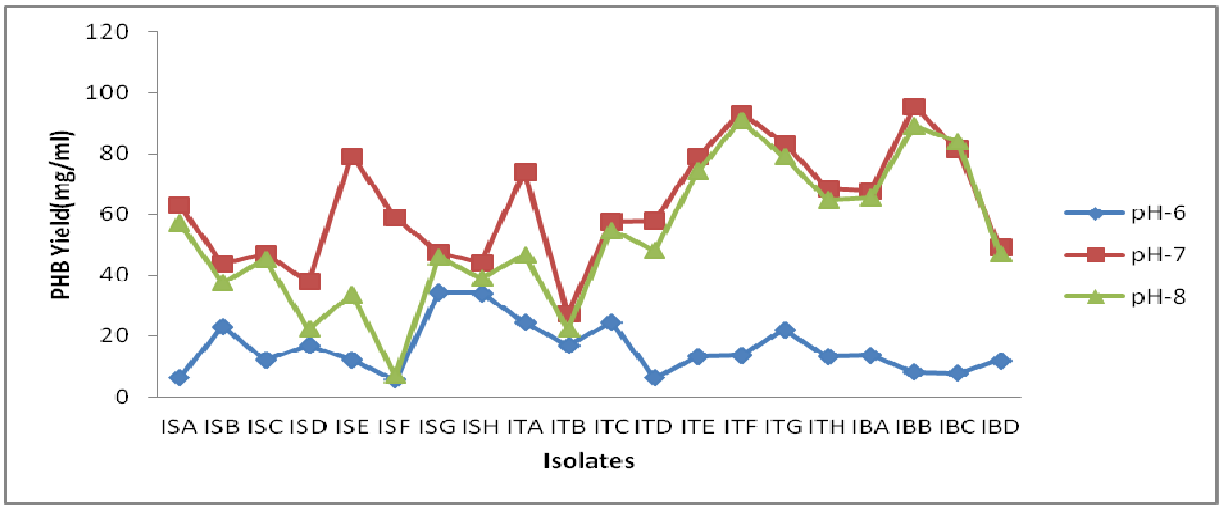


Figure VII

PHB yields by the selected bacterial isolates as influenced by different pH levels

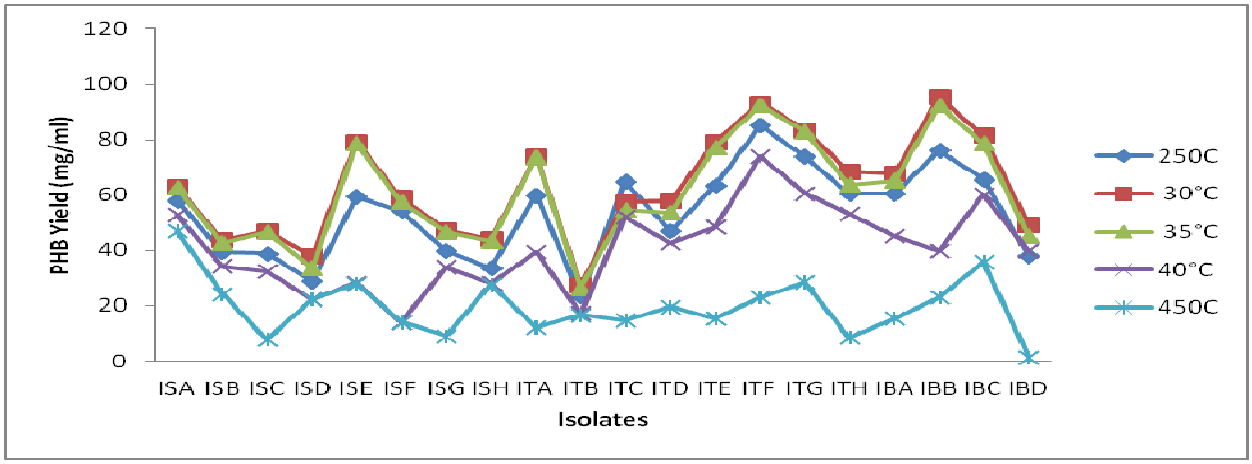


Figure VIII
Effect of different incubation temperatures on PHB production

Table I
Isolation of PHB positive bacteria from soil samples collected from industrial effluent discharge site

S.No.	District	Industrial effluent	No. of isolates	No. of PHB positive isolates (Code assigned)
1	Sonepat	Sugarcane Industry	11	8 (ISA to ISH)
2	Panipat	Thermal Power Plant	13	8 (ITA to ITH)
3	Samalkha	Brewery, Wockhardt	07	4 (IBA to IBD)
Total			**31**	**20**

Table II
PHB production by Sudan Black B positive isolates

S.No.	Industry Sample (District)	PHB Positive Isolate	PHB Yield (mg/ml)	Average PHB Yield (mg/ml)
1.	Sugarcane Industry (Sonipat)	ISA	12.11	**24.31**
2.		ISB	17.32	
3.		ISC	22.94	
4.		ISD	17.89	
5.		ISE	39.69	
6.		ISF	33.45	
7.		ISG	30.93	
8.		ISH	20.21	
9.	Thermal Power Plant (Panipat)	ITA	45.21	**42.81**
10.		ITB	17.22	
11.		ITC	32.84	
12.		ITD	37.94	
13.		ITE	42.99	
14.		ITF	57.78	

15.		**ITG**	**63.35**	
16.		ITH	45.21	
17.	Brewery (Samalkha)	IBA	48.25	
18.		**IBB**	**82.53**	**50.42**
19.		IBC	44.12	
20.		IBD	26.80	
21.	Standard Strain *Cupriavidus necator* (MTCC 1472)		**144.23**	

Table III
Biochemical characterization of PHB positive isolates

S. No	Strain	Gram Staining	Casein	Starch	Catalase	Gelatin	MR	VP	TSI S/B	H2S	Urease	Lactose	Mannitol	Arabinos	Sorbitol	Sucrose	Dextrose	Glucose	Galactose	Identified genera
1.	ISA	+	-	-	+	-	+	+	Y/Y	-	-	-	-	-	-	-	-	-	-	Bacillus sp.
2.	ISB	+	+	-	+	-	-	-	Y/Y	-	+	-	+	+	-	-	-	-	+	Bacillus sp.
3.	ISC	+	-	-	+	-	-	+	Y/Y	-	-	-	-	-	-	-	-	-	-	Bacillus sp.
4.	ISD	+	+	-	+	-	+	+	Y/Y	-	-	-	-	-	-	-	-	-	-	Bacillus sp.
5.	ISE	+	-	-	+	-	-	+	R/Y	-	-	-	-	-	-	-	-	-	-	Bacillus sp.
6.	ISF	+	-	-	+	-	-	+	Y/Y	-	+	-	+	+	-	-	-	-	+	Bacillus sp.
7.	ISG	+	+	-	+	-	+	-	Y/Y	-	-	-	-	-	-	-	+	-	-	Bacillus sp.
8.	ISH	+	-	-	+	-	+	-	Y/Y	-	-	-	-	-	-	-	+	+	-	Bacillus sp.
9.	ITA	+	-	-	+	-	+	-	Y/Y	-	-	-	+	-	-	+	-	+	-	Bacillus sp.
10.	ITB	+	+	-	+	-	+	-	Y/Y	-	-	-	+	-	-	-	-	-	-	Bacillus sp.
11.	ITC	-	-	-	+	-	+	+	R/Y	-	-	-	+	-	-	-	-	+	-	Acinetobacter sp.
12.	ITD	+	+	-	+	-	+	+	Y/Y	-	+	-	+	-	-	-	-	+	-	Bacillus sp.
13.	ITE	+	-	-	+	-	+	-	Y/Y	-	-	-	-	-	-	+	-	+	-	Staphylococcus sp.
14.	ITF	+	-	-	+	-	+	-	Y/Y	-	-	-	-	-	-	+	-	+	-	Bacillus sp.
15.	ITG	+	+	-	+	-	+	-	Y/Y	-	-	-	-	-	-	+	-	+	-	Bacillus sp.
16.	ITH	+	-	-	+	-	+	-	R/Y	-	-	-	-	-	-	-	-	+	-	Staphylococcus sp.
17.	IBA	+	+	-	+	-	+	+	Y/Y	-	-	-	+	+	+	-	-	-	+	Bacillus sp.
18.	IBB	+	+	-	+	-	-	-	Y/Y	-	+	-	+	-	-	-	+	+	-	Staphylococcus sp.
19.	IBC	+	-	-	+	-	+	+	Y/Y	-	-	-	-	-	-	+	-	-	-	Bacillus sp.
20.	IBD	+	-	-	+	-	-	-	Y/Y	-	-	-	-	-	-	+	+	-	-	Staphylococcus sp.

CONCLUSION

The present study was designed to isolate effective polyhydroxybutyrate producing strains and to optimize their culture conditions so as to obtain the maximum PHB yield. After optimization, the PHB yield was found to be enhanced, thus suggesting that the medium composition, specially the C:N ratio greatly influences PHB accumulation. It can therefore be conclusively said that the bacterial PHB yields can be improved by optimizing fermentation conditions at industrial scale. In the present study, two promising isolates (IBB and ITG) were found to accumulate a high level of PHB, at optimized culture conditions; thus, showing a potential for their exploitation in industrial PHB production. The present study has thus provided useful data about the optimized conditions for PHB production that can be utilized for industrial production of

PHB, a fast emerging alternative for non biodegradable plastics.

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REFERENCES

1. Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev, 54: 450-472, (1990).
2. Page WJ. Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics with a Great Future. Canadian Journal of Microbiology, 41(1): 1-3, (1995).
3. Halami PM. Production of polyhydroxyalkanoate from starch by the native isolates *Bacillus cereus* CFR06. World J. of Microbiol. and Biotechnol, 24(6): 805-812, (2008).
4. Wang HH, Li XT, Chen GQ. Production and characterization of homopolymer polyhydroxyheptanoate by a *fad* BA knockout mutant *Pseudomonas putida* KTOY06 derived from *P. putida* KT2442. Process Biochemistry, 44(1): 106-111, (2009).
5. Hyakutake M, Saito Y, Tomizawa S, Mizuno K, Tsuge T. Polyhydroxyalkanoate (PHA) synthesis by class IV PHA synthases employing *Ralstonia eutropha* PH44 as host strain. Bioscience, Biotechnology and Biochemistry, 75(8): 1615-1617, (2011).
6. Rodriguez-Contreras A, Koller M, deSousa Dias MM, Calafell M, Braunegg G, Marques-Calvo MS. Novel poly [(R) - 3-hydroxybutyrate]- producing bacterium isolated from a Bolivian hypersaline lake. Food Technol. and Biotechnol, 51(1): 123-130, (2013).
7. Madison LL, Huisman GW. Metabolic engineering of poly-3-hydroxyalkanoates from DNA to plastic. Microbial Molecular Biology Reviews, 63: 21-53, (1999).
8. Sangkharak K, Prasertsan P. Nutrient optimization for production of polyhydroxyutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electronic Journal of Biotechnology. 11(3): 83-94 (2008).
9. Murray RGE, Doetsch RN, Robinow CF. Determinative and cytological light microscopy. in P. Gerhardt, R.G.E. Murray, W.A. Wood, N.R. Krieg, eds., Manual of Methods for General Microbiology. Washington: American Society for Microbiology, Pp. 21–41 (1994).
10. Mohamed T, Shaaban M, Turky A, Mowafy EI. Production of some biopolymers by some selective Egyptian soil bacterial isolates. J. of Applied Sciences and Research, 8(1): 94-105 (2012).
11. Legat A, Gruber C, Zangger K, Wanner G, Stanlotter H. Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species. Appl. Microbiol. Biotechnol, 87(3): 1119-1127, (2010).
12. Law JH, Slepecky RA. Assay of poly-β-hydroxybutyric acid. J. Bacteriol, 82: 33-36 (1961).
13. Soam A, Singh AK, Singh R, Shahi SK. Optimization of culture conditions for bio-polymer producing *Bacillus mycoides* (WSS2) bacteria from sewage. Current Discovery. 1(1): 27-32, (2012).
14. Gram C. The differential staining of Schizomycetes in tissue sections and in