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Production of Poly- β -hydroxybutyric acid (PHB) by *Bacillus cereus*

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ABSTRACT

One poly- β -hydroxybutyrate producing bacterial from different microbial sources was isolated and characterized for their morphological, biochemical properties. Based on their 16S rDNA, it was identified as *Bacillus cereus* E6. The bacterial strain was screened for PHB production and compared for the intensity of fluorescence using Nile red staining method. PHB production conditions were optimized with different carbon and nitrogen sources, the highest PHB production was observed with sucrose and ammonium sulphate by *B. cereus*, respectively. Regarding incubation time as well as temperature and pH, optimum PHB production conditions were 48 h, 30 °C and 7, respectively. *B. cereus* is capable of accumulating appreciable levels of PHB from glucose, xylose, lactose, whey, molasses, sugar cane bagasse, rice straw hydrolysate when 2% from all substrates were used an alternative carbon for the PHB production. Ammonium sulphate was the best nitrogen source. C/N ratio was also one of the factors that affected the production of PHB. The ratio of C/N that reaches 20:1 was considered the best ratio to produce the highest production of PHB. The present study provide the useful data about the optimized conditions for PHB production by *B. cereus* that can be utilized for industrial production of PHB, a fast emerging alternative of non-biodegradable plastics.

Keywords

poly- β -hydroxybutyric acid, PHB, *Bacillus cereus*

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Introduction

Synthetic polymers - designated as plastics - are applied in a wide range of packing-, household, agricultural, marine and architectural applications. Plastics were developed as light-weight, and durable materials and they have replaced natural resources, such as metals and stones. However, its properties of durability have caused serious problems since plastic waste accumulates in the environment.

The accumulation of abandoned plastics has caused a global environmental problem. Nature usually cannot handle plastic waste, since the majority of plastics are not degraded by microorganisms. At present, about one hundred million tons per year of plastics are produced in the world. With the increase in production, the amount of plastics wastes has raised enormously (Mukai *et al.*, 1995) and increased the costs of solid waste disposal (owing to the

reductions in available landfill space) dramatically. Alternatives to waste disposal such as plastic recycling are quite limited from an economical view point and partially include potential hazards (such as dioxine emission from PVC incineration) (Belal, 2003, 2013 a and b).

Biodegradable plastics opened the way for new waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities. Most of the plastics on the market, claimed to be biodegradable, are based on synthetic and microbial polyesters (Augusta *et al.*, 1993; Witt *et al.*, 2001; Belal 2013 a and b). Polyesters are potentially biodegradable due to the hydrozable ester bonds. In addition, they combine several properties that make them attractive candidates for various industrial applications.

Among various biodegradable polymer materials are Polyhydroxyalkanoates (PHAs) (Page, 1992b; Zhang *et al.*, 2003). This polymer family is made of two major groups- aliphatic and aromatic. Polyhydroxyalkanoate (PHAs) are aliphatic polymer naturally produced via a microbial process on sugar-based medium, where they act as carbon and energy storage material in bacteria. In the 1980s, a new standard for identifying bacteria began to be developed. In the World laboratories, it was shown that phylogenetic relationships of bacteria could be determined by comparing a stable part of the genetic code. (Woese, *et al.*, 1985; Woo, *et al.*, 2003). Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S and the 23S rRNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene (Tortoli, 2003; Harmsen, and

Karch, 2004). The 16S rRNA gene can be compared not only among all bacteria but also with the 16S rRNA gene of archeobacteria and the 18S rRNA gene of eucaryotes (Woese, *et al.*, 1985).

The main member of the PHA family is polyhydroxybutyrate (PHB). PHB is accumulated inside in numerous bacteria under nutrient – limiting conditions with excess carbon. Many references show that number of microorganisms like *Alcaligene eutrophus*, *Azotobacter beijierinckia*, *Pseudomonas Oleovorans*, *Rhizobium* sp. and *Bacillus* sp. produce PHAs as reserve food material. They can be used in different applications such as packaging film and containers, surgical pins and sutures, and bone replacements. PHB degrade naturally and completely to CO₂ and H₂O under natural environment by different microorganisms (Holmes, 1985; Bonartseva *et al.*, 1994; Dahi *et al.*, 1995; Lee, 1996; McCool *et al.*, Yu *et al.*, 2000; Mortia *et al.*, 2001; Mahishi *et al.*, 2003; Das *et al.*, 2005; Philip *et al.*, 2007; Singh and Parmar 2011). The main limitation in commercial production of PHB as bioplastic is its high production cost as compared to that of production of synthetic plastics based on petrochemicals. Low cost agro industrial materials can be used to economize the production.

Many efforts have been devoted to reducing the production costs by isolating bacterial strains capable of growing and producing PHB from inexpensive raw materials and also optimization fermentation conditions for PHB production (Kulpreecha *et al.*, 2009; Belal 2013a). Therefore, the objectives of this study was designed to screen, isolate and characterize of the PHB-producing bacteria and production of PHB on different substrates by using renewable resources.

Materials and Methods

Isolation and selection of PHB-producing strain

Samples of soil were collected from different localities in Kafr El-sheikh Governorate, Egypt. Samples of soil were added as 10 g to 90 ml of sterilized distilled water. The flasks were subjected to shake for 30 min on a rotary shaker (150 rpm) at room temperature. Dilution series were prepared in a glass test tube containing 9 ml distilled water up to $1:10^{-6}$. Ten-fold dilutions were prepared and then 100 μ l was transferred onto sucrose/yeast extract agar (Bormann *et al.*, 1998). The plates were sealed in polyethylene bags and were incubated at 30 °C for 72 h monitored for appearance of colonies. After 48 h incubation at 30 °C, isolated colonies were screened for PHB production. Screening test for PHB (poly- β -hydroxybutyrate) production by different isolates was carried out using Nile red [9-diethylamino-5H-benzo α -phenoxazine-5-one- (sigma)] staining approach (Rehm and Valla 1997; Belal 2013a). Nile red stock solution [0.25 g/ml DMSO (dimethylsulfoxied)], 20 μ l was spread onto sterilized pre-made (sucrose/yeast extract agar media) plates to reach a final concentrations of 0.5 μ g Nile red /ml medium.

After inoculation, the plates were incubated at 30 °C for 48 hrs subsequently; colonies with pinkish pigment indicated PHB production isolates were also exposed to ultraviolet light (312 nm) to detect their lightness, after that these lighted plates were recorded positives PHB production and these isolates were selected for the subsequent experiments (Spiekermann *et al.*, 1999). All experiments were performed in triplicates. The culture was maintained on sucrose/yeast extract agar and stored at 4°C.

Extraction and estimation of PHB production efficiency

Conical flask (500 ml) containing 100 ml of sucrose/yeast extract medium were sterilized at 121 °C for 20 min and inoculated with 1ml of inoculum containing 10^8 cfu / ml (sucrose/yeast extract medium, 10^8 cfu / ml, incubated at 30 °C and 150 rpm for 3 days). The inoculated flasks were incubated at 30 °C and 150 rpm for 48 h. PHB was determined by the described below method. The efficient isolate was selected according to the highest PHB productivity. Three replicates from each treatment were used.

PHB dry weight assay

Cells were collected by centrifugation at 10 000 rpm for 10 min from 1 to 5 ml samples of the culture and the pellet was re suspended in 10 ml of sodium hypochlorite reagent (pH 10.0-10.5 NaOCl content 5.25%-5.5%) according to the method recommended by Williamson and Wilkinson, (1958); Hahn, (1994) and Belal (2013a). After 1 h at 37 °C the reaction mixture was centrifuged at 5000 rpm for 10 min.

The whole mixture was again centrifuged at 10 000 rpm for 10 min and the supernatant was discarded. The solid pellet was washed successively with 1 ml portions of water, alcohol and acetone, the final pellet was dissolved in chloroform, while the insoluble residue was discarded. Finally, the chloroform was evaporated at room temperature and the polymer was dried at 105 °C for 2 h until constant weight and then weighed. Dry weight of extracted PHB was estimated as g/L. The powdery mass along the test tube wall was collected. Dry weight of the biodegradable polymer and percentage (w/w) of it against cell dry weight was measured. Residual biomass was

estimated as the difference between dry cell weight and dry weight of PHB.

PHB spectrophotometer assay

A sample containing polymer in chloroform is transferred to a clean test tube. The chloroform is evaporated and 10 ml of concentrated H₂SO₄ were added. The tube is capped with a glass marble and heated for 10 min at 100 °C in a water bath. The solution is cooled and after thorough mixing a sample is transferred to a silica cuvette and the absorbance at 235 nm in a UV spectrophotometer is measured against a sulfuric acid blank. A standard curve was established with PHB (Sigma Aldrich) concentrations according to Slepecky and Law, 1960b; Kuniko *et al.*, 1988 and Belal 2013a.

Cell dry weight estimation

After incubation time, culture medium was collected and centrifuged at 10,000 rpm for 15min. Supernatant was discarded and the cell pellet was washed twice in deionized water and recovered for 10 min at 10000 rpm at 4°C. The cell pellet was dried at 100°C for 24 hr until constant weight then the total bacterial cell dry weight was determined as g/L (Kuniko *et al.*, 1988, Ishizaki *et al.*, 1991; Belal 2013; Hawas *et al.*, 2016).

Identification

The efficient selected PHB producing bacterial isolate was identified depending morphological and physiological characteristics as described by Parry *et al.*, (1983) and 16S rDNA sequencing as follow:

16S rRNA sequence determination

The amplified product of approximately 1254 bp (1,254 bp for nested PCR) was

carried out according to Claudio, *et al.*, (2002). Sequencing was performed using a big dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA). Sequencing products were purified by using Centri-Sep™ Columns (Princeton Separations, Adelphia, NJ) and were resolved on an Applied BioSystems model 3100 automated DNA sequencing system (Applied BioSystems).

The evolutionary history was inferred using the Minimum Evolution method according to Rzhetsky and Nei, (1992). The optimal tree with the sum of branch length = 0.67162741 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method according to Tamura *et al.*, (2004), and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) and a search level of the Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 765 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2013).

Optimization of cultural conditions for maximum PHB production

Different media

The different media medium 1 (Sucrose / yeast extract broth medium (Bormann *et al.*, 1998), medium 2 (nutrient broth medium (Atlas, 1997), medium 3 (Bänziger and

Tobler 2001), medium 4 (synthetic medium, Burdman *et al.*, 1998) were used. 100 ml of each previous media were sterilized at 121 °C for 20 min and inoculated with 1ml of inoculum containing 10^8 cfu / ml (sucrose/yeast extract medium, 10^8 cfu / ml, incubated at 30°C and 150 rpm for 3 days) of *B. cereus*.

The inoculated flasks were incubated at 30 °C at 150 rpm for 48 h. PHB was determined by the described method to select the optimum medium. Three replicates from each treatment were used.

pH and temperature optimization

To determine the optimum pH, experiments were carried out at pH (6, 6.5, 7, 7.5 and 8). Cultures were incubated on a rotary shaker at 30 °C and 150 rpm for 48 hrs. To determine the effect of temperature sucrose / yeast extract medium with pH 7 was incubated at 25, 30, 35, 40, and 45 °C under 150 rpm for 48 hrs. After 48 hrs, PHB yields were extraction and quantified as described before. Three replicates from each treatment were used.

Optimization of incubation time

To determine the effect of incubation period, the inoculated sucrose / yeast extract liquid medium were incubated at 30 °C and pH7 at 150 rpm under different incubation period (24, 48, and 72 hrs). PHB yields were extraction and quantified as described before. Three replicates from each treatment were used.

Optimization of carbon and nitrogen sources for PHB production

To detect the effect of carbon sources, sucrose / yeast extract broth medium was prepared and supplemented with different

concentrations of carbon sources (1 % and 2 %) at the optimum culture conditions (pH 7 and 30 °C). The carbon sources included sucrose, glucose, xylose, lactose, starch, whey (industrial dairy products), and agricultural residues such as molasses, sugar cane bagasse and rice straw hydrolysate (Rice straw and sugar cane bagasse hydrolysates were prepared and reducing sugars were determined as described methods by Miller, (1959), Santimano *et al.*, (2009) and Ghate *et al.*, (2011).

Effect of different nitrogen sources on PHB production

To detect the effect of nitrogen sources sucrose / yeast extract medium was prepared with the optimum carbon source and supplemented with different concentrations of nitrogen (0.5 and 1g/L) at pH 7 and 30 °C for 48 hrs. The nitrogen sources included ammonium sulphate (NH₄)₂SO₄, ammonium chloride (NH₄Cl), ammonium nitrate, yeast extract and proteose peptone.

Ammonium sulphate (NH₄)₂SO₄ as best N source was optimized with different concentration. Three concentrations (0.5, 1 and 1.5 g/L) were used. After 48 hrs, PHB yields were extraction and quantified as described before. Three replicates from each treatment were used.

Effect of different C:N ratios on PHB

The best concentration from carbon source (sucrose 2%) and N source (ammonium sulphate 1g/L⁻¹) was used. In addition to, different C:N ratios such as 15:1, 20:1 and 25:1 using the best C and N sources were done after inoculation and incubated on a rotary shaker (150 rpm) at 30°C. After 48 hrs, PHB yields were extraction and quantified as described before. Three replicates from each treatment were used.

Statistical analysis

Data were calculated as mean \pm standard deviation (SD) and analyzed using analysis of variance (ANOVA). Probability of 0.05 or less was considered significant. The statistical package of Costat Program (1986) was used for all chemometric calculations.

Results and Discussion

Isolation and screening of PHB producing bacteria

The present study was carried out to isolate several bacterial isolates from soil which collected from different localities in Kafr El-Sheikh governorate, Egypt. Ten bacterial isolates obtained from the preliminary screening following the viable colony staining technique were subjected to quantify of PHB production. The bacteria were initially screened for the PHB production in sucrose/yeast extract agar medium and the ability to synthesize PHB granules was confirmed using Nile red staining of PHB granules in the intracellular environment of the isolated bacteria. Colonies with pinkish pigment indicated PHB production isolates were exposed to ultraviolet light (312 nm) to detect the accumulation of PHB according to the lighted plates were recorded positives, after that these isolates were picked up and purified by sub-cultured on the same media.

Based on the intensity of the fluorescence observed in the staining methods the potential PHB producers were identified. Colonies of PHB-accumulating strains should fluorescence under ultraviolet irradiation when the cells were cultivated under conditions permitting PHB accumulation (Figure 1). The results showed that the positive isolates exhibited fluorescence. Whereas the others

corresponding isolates showed no signals with the Nile-red test. This finding is considered an additional evidence for the specificity of the Nile-red staining approach to screen new bacterial isolates for PHAs production. Therefore, the direct addition of these dyes to the medium should provide a tool to discriminate between PHB negative and PHB-positive strains without killing the cells (McCool, 1996; Spiekermann *et al.*, 1999; Belal, 2013a; Hawas *et al.*, 2016).

All isolates subjected for screening by Nile-red staining approach, also quantitative with spectrophotometric assay using H₂SO₄ according to (Slepecky and Law, 1960b; Kuniko *et al.*, 1988). It was established that the Nile-red stain emitted strongly positive fluorescence signals only with a hydrophobic compounds like PHAs and lipids. Nile-red intended to show any lipid particles inside the cells and it did not react with any tissue constituent except by solution and could be detected by fluorescence spectroscopy or flow cytometry, my results are in agreement with (Degelau *et al.*, 1995; Gorenflo *et al.*, 1999; Spiekermann *et al.*, 1999, Lakshmi *et al.*, 2012). The positive bacteria were assigned with the code number.

Based on the PHB yields, the obtained results in Table 1 shows that the content of PHB in the isolated strains. The amount of PHB in strains was 0.11 – 3.6 g/l, and the percentage of PHB in these cells was between 12.2 and 64.3% of dry cell weight. The highest PHB productivity was in soil bacteria designated as E6, the lowest PHB productivity was found in soil bacteria designated E10 (12.2 %). It was found that a significant relationship existed between dry cell weight and PHB production.

Out of 10 positive isolates, E6 was finally selected as promising isolates in PHB

production for further studies. Production of PHB has most commonly been studied on microorganisms belonging to the genera *Alcaligenes*, *Azotobacter*, *Bacillus* and *Pseudomonas* (Singh and Parmar, 2011).

PHB is a carbon storage polymer widely distributed among prokaryotes including *Rhizobium*, *Bradyrhizobium* and nodule bacteroides (Nair *et al.*, 1993). In recent years, PHB and other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastics (Lee, 1996). Many nitrogen-fixing microorganisms synthesize PHB. According to Tombolini and Nuti, (1989), the content of this polymer in rhizobia ranges from 30 to 55% of dry cell weight. Bonartseva *et al.*, (1994) tested the capacity for PHB production in active and less active strains of *Rhizobium phaseoli*, *R. meliloti* and *R. trifolii* during growth on media with different carbon and nitrogen sources. It was found that PHB synthesis can be selectively induced either in active or less active *Rhizobium* strains by sources of carbon and nitrogen. They reported that the less active strain of *R. phaseoli* 680 was a promising producer of PHB, and the PHB content in cells of this strain was up to 65 % of dry cell weight during growth on a medium with sucrose and nitrate; the PHB content was much lower when organic acids were used. Many investigators reported the ability of biodegradable polymer production by various members of the genera *Pseudomonas* (Aremu *et al.*, 2010).

Identification of the efficient of PHB production isolate

Results in Figure (2) showed that this bacterial strain (E6) was identified according to morphological, physiological as well as using analysis of 16S rDNA. The isolated strain appeared as off-white, circular, smooth, large colony. Microscopically it appeared as rod shaped

gram positive spore forming *Bacillus*. The presence of endospores and the positive test for catalase activity for the organism indicated that the isolate could be a *Bacillus* species.

The molecular phylogeny of sample was determined by analyzing 16S rDNA gene sequences. According to the 16S rDNA analysis, the phylogenetic tree of the PHB producing bacteria E6 and related bacterial species based on the 16S rDNA sequence is provided in Fig. 2. It can be clearly seen that the *Bacillus* sp. E6 as PHB production bacteria was included in the genus *Bacillus* and closely related to the species *cereus*. It showed the highest sequence similarities with *Bacillus cereus* strain ATCC 10987 (98 %). Earlier such studies also reported isolation and characterization of PHB from *Bacillus* spp. isolated from natural habitats (Thirumala *et al.*, 2010; Aarthi and Ramana 2011; Babruwad *et al.*, 2015).

Optimization of cultural conditions for PHB production

Effect of different fermentation media on PHB production

The results presented in Table (2) showed a maximum yield of PHB on medium 1 by *B. cereus*. The maximum percentage of PHB was 69.8, 57.1, 38.2 and 41.4 on medium 1, 2, 3 and 4 by *B. cereus*, respectively. The lowest percentage of PHB was on medium 3. The highest PHB production and percentage productivity were obtained on medium 1 and followed by the other media. Medium 1 (Sucrose yeast extract medium) was selected for the subsequent investigation.

Effect of different pH values on PHB production

Data presented in Table 3 showed that, out of the different pH of medium, pH7 was

found to be optimum for maximum PHB production by *B. cereus*. The highest PHB production of 3.3 g/L culture was produced by *B. cereus* at pH 7.0. The strain was found to produce lower yields showing at pH 6, 6.5, 7.5 as well as 8 and pH 7.0 was found as the best pH for PHB production. As shown in Table 3 the maximum PHB production occurred at a pH range 7.0 at 30°C after 48 h of incubation. The obtained results were in agreement with Aslim *et al.*, (2002) who observed that the PHB in *Rhizobium* strain grown on yeast extract mannitol broth adjusted to pH 7.0, the amount of PHB was 0.01 to 0.5 g/L culture and the percentage of PHB in these cells was between 1.38 and 40 percent of cell dry weight. Tavernler *et al.*, (1997) also investigated the effect of different nitrogen, carbon sources and different pH values on the production of PHB by strains of *Rhizobium meliloti*. They reported that these strains showed higher PHB content at pH 7.0. These findings are in agreement our previous study (Belal 2013a and Hawas *et al.*, 2016).

Data presented in Table 4 showed that, incubation at 30°C was found to be optimum and has produced 3.6 g/L after 48 h of incubation at pH 7. The highest PHB production of 3.6 g/L culture was produced by *B. cereus* at 30 °C. The strain was found to produce lower yields showing at 20 and 40 °C. As shown in Table 4 the maximum PHB production occurred at 30 °C after 48 h of incubation at a pH 7. These results supported by (Sangkharak and Prasertsan, 2007). Pozo *et al.*, (2002) studied effects of culture conditions on PHB production by *Azotobacter* sp. and showed that growth conditions including pH, temperature plays an important role in the production rate of PHB. The PHA production by a particular strain also related to its biomass. As the biomass increases the bacteria also starts accumulating PHB and produce maximum

PHB when its biomass is at its peak level and PHB production is slowed down as the biomass is dropped because at this phase of the growth all the nutrients are depleted leading to decrease in PHB content.

Table 5 shows the effect of incubation time on PHB production by *B. cereus*. The PHB yields increased with time dependent manner and highest yield 3.6 g/L was obtained after 48 h for growth of *B. cereus*. The PHB yield decreased to 2.1 at 72 h. According to Bonartseva *et al.*, (1994) the results confirmed the observed maximal PHB accumulation at 48 h. After 48 h there was a decrease in PHB yield and an increase in the viscosity of the medium at 72, 96 and 120 h. After 48 h, then unfavorable conditions of the medium caused the decrease in PHB yield, because the increase in medium viscosity accompany exopolysaccharides production resulting in oxygen transfer limitation, caused the decrease in PHB synthesis, (Stam *et al.*, 1986a; Tombolini and Nuti, 1989; Encarnacion *et al.*, 1995). The yield decreased at 72 and 120 h although dry cell weight increased at 120 h, the decrease of PHB indicates that the bacteria used PHB as a source of carbon, causing unsuitable conditions due to inadequate nitrogen and carbon sources in the medium. The highest production of PHB was after 48 h of incubation. Correspondingly there was decrease in glucose consumption in the course of time. Also the concentration of PHB decreased after 48 h. This reduction in PHB production after 72 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production.

Effect of different carbon sources on PHB production by *B. cereus*

The data illustrated in (Table 6) depict the effects of different concentrations from

carbon sources on PHB yield. This shows that the ability of the bacterium to utilize different complex carbon sources substrates is variable and is dependent on several factors like nature of the substrate used and the type of enzyme produced.

In the present study, *B. cereus* had shown preference for carbon sources like sucrose, glucose, xylose, lactose, molasses, starch, whey, sugar cane bagasse and rice straw hydrolysate. In general, there was an increasing of PHB production with an increase in carbon sources concentration from 1 to 2 % by *B. cereus*. The highest PHB productivity was observed by *B. cereus* growing on sucrose being 1.3 – 3.7 g/L (44.8 – 67.3%) and is dependent on sucrose concentration. *B. cereus* utilizes carbon sources and produced PHB in range from 1.3 – 3.7 g/L. The next promising C sources were glucose, molasses, lactose, whey, xylose, starch and followed by the raw materials such as sugar cane bagasse and rice straw hydrolysate.

Among the different carbon sources tested to evaluate their effects on PHB yield, sucrose was found to be the best carbon source for *B. cereus*. This was followed by glucose and all the carbon sources. The lowest PHB production was obtained with rice straw hydrolysate on dry cell weight basis of *B. cereus*. It was shown that the highest concentration from raw materials enhanced PHB synthesis in *B. cereus*.

Poly-β-hydroxybutyric acid (PHB) was produced from glucose (ranged from 1.3 to 2.3 g/L), xylose (ranged from 0.2 to 0.6 g/L), lactose (ranged from 0.5 to 0.8 g/L), whey (ranged from 0.7 to 1.4 g/L), molasses (ranged from 0.5 to 1.1 g/L), starch (ranged from 0.3 to 0.5), sugar cane bagasse (ranged from 0.2 to 0.3) and rice straw hydrolysate (ranged from 0.15 to 0.19 g/L) by *B. cereus*, respectively.

In order to Produce PHB more economically, the use of cheap and easily available raw materials such as whey is desirable. Few reports are available on the use of whey as a resource for PHB production. Cheese whey is produced in very large volumes worldwide (Siso, 1996). Much of this is discarded because it has limited use. Additionally, whey requires expensive treatment prior to its disposal and is therefore attractive as a raw material for PHB production. *B. cereus* is capable of accumulating appreciable level of PHB from lactose and whey, therefore offers much potential for economic production of PHB from such raw materials. A strain of poly-β-hydroxybutyrate) (PHB)-accumulating bacterium was isolated from the soil in Alaska of USA, identified as *Pseudomonas fluorescens*, and designated as strain A2a5. The organism grew at temperatures below 30 °C, and accumulated a large amount of granules in its cells when it was cultured in the sugarcane liquor medium (Jiang *et al.*, 2008). Moreover, our results suggest that production of PHB from hemicellulosic hydrolysates and cheese whey by *P. cepacia* may be possible (Young *et al.*, 1994).

Whey permeates from dairy industry was hydrolyzed enzymatically to cleave its main carbon source, lactose, to glucose and galactose. The hydrolysis products were chosen as carbon sources for the production of poly-3-hydroxybutyric acid (PHB) by *Pseudomonas hydrogenovora*. In shaking flask experiments, the utilization of whey permeate as a cheap substrate was compared to the utilization of pure glucose and galactose for bacterial growth under balanced conditions as well as for the production of PHB under nitrogen limitation. The strain *P. hydrogenovora* DSM 1749 is able to cometabolize glucose and galactose from lactose-hydrolyzed whey permeate to produce PHB (Koller *et al.*, 2008)

This indicates that for an organism to efficiently use whey as substrate, it must be able to metabolize the lactose as its carbon source. Such organisms should produce sufficient lactose hydrolyzing enzymes (β -galactosidase) to hydrolyze the lactose to its monomers (Khanafari and Sepahei, 2007). There are very few reports of PHA production from whey by indigenous microorganisms. Young *et al.*, (1994) demonstrated that PHB can be produced from xylose and lactose by using the wild-type microorganism *Pseudomonas cepacia* ATCC 1775. Gerhart, (2008) reported PHA production from whey permeate using *Pseudomonas hydrogenovora*. Yellore and Desai, (1998) and Nath *et al.*, (2005) reported production of poly (β - hydroxybutyrate) (PHB) from disaccharides like lactose and sucrose by *Methylobacterium* sp. ZP24 and from bagasse (Yu and Heiko, 2008) as well as from food wastes (Yu *et al.*, 1998).

Molasses, which is a common industrial by-product, is much cheaper than glucose, and has been extensively used as a carbon source in PHB production by *Azotobacter vinelandii* (Page, 1992b). Page, (1992) reported that the strains of *Rhizobium* produced less PHB in yeast extract mannitol (YEM) broth media with different carbon (glucose, sucrose and arabinose). Reports on using molasses as the sole carbon source to produce PHB by *Alcaligenes eutrophus* have also appeared in the literature in recent years (Beaulieu *et al.*, 1995). These findings are in agreement our previous study by *Rhizobium elti* E1 and *Pseudomonas stutzeri* (Belal 2013a).

Effect of different nitrogen sources on PHB production by *B. cereus*

Data pertaining to PHB yields produced by *B. cereus* in presence of different concentrations from N sources and the best carbon source (sucrose 2%) are presented in

Table 7. This shows that the ability of the bacterium to utilize different nitrogen sources substrates is variable and is dependent on nature of the substrate used. In general, there was an increasing of PHB production with an increase in nitrogen sources concentration from 0.5 to 1g/L by *B. cereus*. *B. cereus* produced a mean PHB in range from 0.6 – 3.9 g/L. Among different N sources, ammonium sulphate was found to be the best N source. It produced a mean PHB of 2.9 - 3.9 g/L by *B. cereus*. The next promising N sources were ammonium chloride with (1.4 -1.9 g/L), ammonium nitrate with (1.1 – 1.4 g/L), proteose peptone (0.6 – 0.9 g/L) PHB yields. The highest PHB was obtained in ammonium sulphate followed by ammonium chloride. Proteose peptone was found to be the least supporter of PHB production.

These results are in agreement with the results obtained by Khanna and Srivastav, (2005), Shaaban *et al.*, (2012) who also observed the highest PHB production by *R. eutropha* or *Stenotrophomonas* species and *Pseudomonas* species on MSM medium supplemented with ammonium sulphate. Mulchandani *et al.*, (1989) and Raje and Srivastav, (1998) also worked on the accumulation of PHB by *A. eutrophus* with different salts of ammonium.

Effect of different concentrations of ammonium sulphate on PHB production by *B. cereus*

PHB yields produced by *B. cereus* when grown on different concentrations of the best N source (ammonium sulphate) in presence of the best carbon source (sucrose 2%) are presented in Table 8. *B. cereus* produced a mean PHB yield of 4.2 g/L. Out of three concentrations of ammonium sulphate, it was found that of ammonium sulphate, at 1 g/L supported the highest PHB production when compared to other levels. In general, there was an increasing of PHB production

with an increase in ammonium sulphate concentration from 0.5 g/L to 1.0 g/L. It was found that *B. cereus* was found to produce the highest PHB yield of 4.2 g/L at 1.0 g/L concentration. The presence of higher concentration of nitrogen (1.5 g/L) in the culture medium was inhibitor for the accumulation of PHB. Hence, the concentration of the best nitrogen source needs to be optimized. From the present studies, it was revealed that ammonium sulphate at 1 g/L was the optimum level for PHB production by *B. cereus*. When ammonium sulphate concentration was increased from 0.5 g/L to 1 g/L, the PHB accumulation was also increased. But, at 1.5g/L the PHB accumulation was decreased. This may be due to the absence of nitrogen stress condition required for accumulation of PHB. The effectiveness of ammonium sulphate 1g/L in enhancing PHB production is in accordance with Khanna and Srivastav, (2005) and Shaaban *et al.*, (2012) who found that this level produced the highest PHB by *R. eutropha* and *Stenotrophomonas* species and *Pseudomonas* species, respectively .

Effect of different C:N ratios on PHB production *B. cereus*

Different C:N ratios were maintained using

the best carbon and nitrogen sources in sucrose/yeast extract as well as their effects on PHB production were studied. The C:N ratios were adjusted to 15:1, 20:1 and 25:1 using sucrose and ammonium sulphate as C and N sources for *B. cereus*. The data are presented in Table 9 showed that, *B. cereus* was found to be significantly higher PHB producer mean where the PHB of 4.6 g/L culture, respectively. Amongst the different C:N ratios tested, 20:1 was found to be best C:N ratio supporting the highest PHB production. In all the tested strains, there was an increasing PHB production with an increase of C:N ratios up to 20:1 and tapering therefore. The highest yield of PHB (4.6 g/L culture) was by *B. cereus* at 20:1.

Under normal conditions, bacteria synthesize their cell materials like proteins and grow. But, in nutrient limiting conditions, bacteria may shift their protein synthesis to PHB synthesis for survival. At 25:1 C:N ratio, PHB yields were significantly lower by *B. cereus* when compared to those at 20:1. As the carbon content increased in the media keeping N as constant, up to certain limit (i.e., 20:1) PHB accumulation was increased and there off (25:1), it showed a decline.

Table.1 Screening of PHB production by the different bacterial isolates

Isolates	Cell dry weight (g / L)	PHB (g / L)	% PHB yield
E1	2.3 ± 0.1	0.9± 0.04	39.1± 0.11
E2	1.1± 0.13	0.2± 0.11	18.2± 0.12
E3	1.3± 0.14	0.2± 0.11	15.4± 0.15
E4	1.2± 0.14	0.3± 0.11	25± 0.17
E5	0.7± 0.11	0.21± 0.1	30± 0.13
E6	5.6± 0.1	3.6± 0.1	64.3± 0.21
E7	2.3± 0.15	0.7± 0.2	30. 4± 0.3
E8	1.9± 0.31	0.62± 0.21	32.6± 0.25
E9	1.2± 0.35	0.4± 0.21	33.3± 0.43
E10	0.9± 0.13	0.11± 0.03	12.2± 0.13

Table.2 Effect of different fermentation media on PHB production.

Medium	Cell dry weight (g / L)	PHB (g / L)	% PHB yield
Medium 1	5.3± 0.2	3.7± 0.21	69.8± 0.13
Medium 2	4.2± 0.23	2.4± 0.3	57.1± 0.3
Medium 3	2.7± 0.13	0.9 ± 0.21	33.3± 0.2
Medium 4	2.9± 0.35	1.2± 0.13	41.4± 0.22

Table.3 Effect of pH on PHB production by production by *B. cereus*

pH	Cell dry weight (g / L)	PHB (g / L)	% PHB yield
6	1.5± 0.31	0.5± 0.11	33.3± 3.1
6.5	2.4± 0.21	1.1± 0.16	45.8±2.4
7	5.1± 0.13	3.3± 0.11	64.7± 1.1
7.5	3.4± 0.24	1.4± 0.13	41.2± 2.3
8	1.3± 0.17	0.4± 0.21	30.8± 3.1

Table.4 Effect of temperature on PHB production by *B. cereus*

Temperature	Cell dry weight (g / L)	PHB (g / L)	% PHB yield
20	1.2± 0.3	0.4± 0.31	33.3± 3.3
30	5.4± 0.12	3.6± 0.13	66.7± 1.1
40	1.1± 0.19	0.34± 0.15	30.9± 2.7

Table.5 Effect of incubation time on PHB production by *B. cereus*

Incubation time (hr)	Cell dry weight (g / L)	PHB (g / L)	% PHB yield
24	3.1± 0.31	1.4± 0.23	45.2± 0.41
48	5.4± 0.22	3.6± 0.11	66.7± 0.22
72	4.5± 0.21	2.1± 0.31	46.7± 0.51

Table.6 Effect of different carbon source on PHB production by *B. cereus*

Carbon source	Carbon source concentrations					
	1 %			2 %		
	Cell dry weight (g/L)	PHB (g/L)	% PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
Sucrose	2.9± 0.12	1.3± 0.1	44.8±1.2	5.5± 0.2	3.7± 0.07	67.3± 0.22
Glucose	2.8± 0.03	1.3± 0.03	46.4±1.3	4.4± 0.3	2.3± 0.11	52.3± 3.1
Xylose	1.2± 0.2	0.2± 0.01	16.7±1.2	1.5±0.23	0.6± 0.02	31.3± 2.1
Lactose	1.3± 0.11	0.5± 0.01	38.5±2.1	1.8±0.15	0.8± 0.31	44.4± 2.1
Molasses	1.6± 0.21	0.5± 0.1	37.5±1.3	2.3±0.07	1.1± 0.05	47.8± 1.3
Starch	1.5± 0.01	0.3± 0.1	20± 1.2	1.8±0.12	0.5± 0.11	27± 2.3
Whey	2.5± 0.3	0.7± 0.01	28±0.5	3.9± 0.3	1.4± 0.03	35.9± 1.2
Sugar cane bagasse	0.98± 0.2	0.2± 0.1	20.4±1.1	1.3±0.41	0.3± 0.15	23.1± 1.2
Rice straw hydrolysate	0.92± 0.1	0.15±0.01	16.3±1.1	1.1± 0.2	0.19± 0.03	17.3± 2.3

Table.7 Effect of different nitrogen source on PHB production by *B. cereus*

Nitrogen source	Nitrogen source concentrations					
	0.5g/L			1g/L		
	Cell dry weight (g/L)	PHB (g/L)	% PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
Ammonium sulphate	4.2± 0.1	2.9± 0.12	69.1±1.03	5.1±0.13	3.9± 0.1	76.5± 0.3
Ammonium chloride	3.3± 0.2	1.4± 0.2	42.4±2.1	3.7± 0.3	1.9±0.25	51.4± 2.1
Ammonium nitrate	2.6± 0.21	1.1± 0.31	42.3±3.2	3± 0.4	1.4±0.31	46.7± 2.1
Proteose peptone	2.8± 0.2	0.6± 0.11	21.4±1.1	3.3±0.3	0.9±0.13	27.3± 2.3

Table.8 Influence of different ammonium sulphate concentrations on PHB production by *B. cereus*

Ammonium sulphate concentrations								
0.5g/L			1g/L			1.5g/L		
Cell dry weight (g/L)	PHB (g/L)	%PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
3.7 ± 0.21	2.4 ±0.31	64.9 ±3.1	5.4 ± 0.11	4.2 ±0.13	77.8 ±0.11	4.2 ±0.14	2.9 ±0.11	69.1 ±0.21

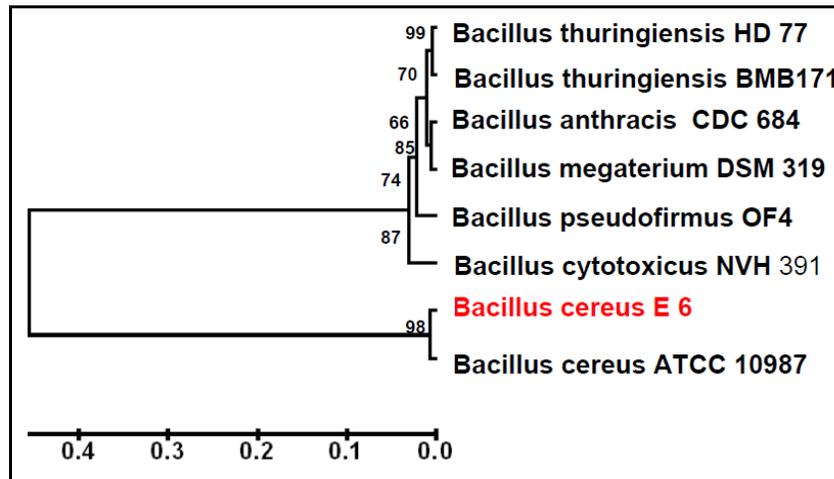
Table.9 Effect of different C:N ratios of medium on PHB yields by *B. cereus*

C:N ratios								
15:1			20:1			25:1		
Cell dry weight (g/L)	PHB (g/L)	% PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
4.1 ± 0.2	2.6 ±0.31	63.4 ±3.2	5.6 ±0.11	4.6 ±0.1	82.1 ±1.3	4.9 ±0.3	3.4 ±0.23	69.4 ±2.3

Fig.1 Fluorescence of PHB granules using Nile red staining where: A) The isolate that did not yield signals with the Nile red staining assay. B) Nile red staining assay showing the PHB positive (strong fluorescence).



Fig.2 Phylogenetic tree showing the diversity of bacterial 16 S gene sequences from *Bacillus cereus* (E6). Phylogenetic trees were generated using parsimony, neighbor-joining and maximum likelihood analysis



This was probably due to the substrate inhibition. Similar observations were made by other scientists. Belfaos *et al.*, (1995) and Shaaban *et al.*, (2012) reported that glucose and ammonium ions were inhibitory at certain levels, which affect the specific growth rate and PHB production. Inhibition by ammonium ions Heinzle and Lafferty, (1980) and substrate inhibition by carbon source Lee and Yoo, (1991) on PHB production have been reported.

In conclusion, it can be concluding that optimum culture conditions for PHB production by *B. cereus*. The existence of carbon source where the sugar of sucrose was considered the best carbon source to produce PHB compared to the other carbon sources by *B. cereus*. In the present work, a low cost of raw material as sugarcane, molasses, whey and rice straw hydrolysate could improve the economics of the process and obtained high PHB production when 2%

from all substrates were used an alternative carbon for the PHB production. The presence of nitrogen source where ammonium sulphate was the best source of nitrogen that helps to produce PHB compared the other concentration of the studied concentrations of ammonium sulphate. Regarding incubation time and temperature and pH, optimum PHB production conditions were 48 h, 30°C and 7.0, respectively. C/N ratio was also one of the factors that affected the production of PHB by *B. cereus*. The ratio of C/N that reaches 20:1 was considered the best ratio to produce the highest production of PHB. Thus by utilizing the optimum culture conditions we can solve the problems of high cost of PHB production.

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