

## Research Article

### Production of Poly- $\beta$ -Hydroxybutyric Acid (PHB) by *Rhizobium elti* and *Pseudomonas stutzeri*

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**Abstract:** The amount of chemosynthetic plastic waste increases every year and exact time for its degradation is unknown. Two poly- $\beta$ -hydroxybutyrate producing bacteria from different microbial sources were isolated and characterized for their morphological, biochemical properties. Based on their 16S rDNA, they were identified as *Rhizobium elti* E1 and *Pseudomonas stutzeri* E114. The bacterial strains were screened for PHB production and compared for the intensity of fluorescence using Nile red staining methods. PHB production conditions were optimized with different carbon and nitrogen sources, the highest PHB production was observed with mannitol, sucrose and ammonium sulphate by *R. elti* and *P. stutzeri*, respectively. Regarding incubation time as well as temperature and pH, optimum PHB production conditions were 48 h, 30°C and 7, respectively. *R. elti* and *P. stutzeri* are 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 highest yield of PHB was done by *P. stutzeri* more than *R. elti*. The present study provide the useful data about the optimized conditions for PHB production by *R. elti* and *P. stutzeri* that can be utilized for industrial production of PHB, a fast emerging alternative of non biodegradable plastics.

**Keywords:** PHB, *Rhizobium elti*, polyhydroxybutyrate, *Pseudomonas stutzeri*

## 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 and Doi, 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).

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). 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. 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 beijerinckia*, *Pseudomonas oleovorans*, *Rhizobium* sp., etc., 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 CO<sub>2</sub> and H<sub>2</sub>O under natural environment by different microorganisms (Holmes, 1985; Bonartseva *et al.*, 1994; Dahi *et al.*, 1995; Lee, 1996; Yu *et al.*, 2000; Morita *et al.*, 2001; Mahishi *et al.*, 2003; Das *et al.*, 2005; Philip *et al.*, 2007). 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. Therefore, the present investigation was designed to isolate and characterize of the PHB-producing bacteria from different microbial sources and production of PHB on different substrates by using renewable resources.

## MATERIALS AND METHODS

**Medium:** Sucrose/Yeast extracts (Bormann *et al.*, 1998) and yeast extract mannitol (Vincent, 1970) were used through this study.

**Sources of microorganism:** Rhizosphere soil and active root nodules of healthy bean plants samples were collected from different localities in Elbehira governorate, Egypt in a sterile polythene bags and brought to the laboratory to use their a source of microorganism. Soil samples were subjected to direct dilution and plating on sucrose/yeast extract agar medium. The both medium was supplemented with Nile red. The plates were incubated at 30°C for 48 h. Various colonies with pinkish pigment were selected for further studies.

Rhizobial isolate was isolated from active nodules initiated on healthy bean plants on yeast extract mannitol agar according to Vincent (1970). *Rhizobium*-like colonies were subjected to different cultural, biochemical, genetical and plant inoculation test for identification according to (Somasegaran and Hoben, 1985).

**Screening of PHB producing bacteria via viable-colony staining:** 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). Nile red stock solution (0.25 g/mL DMSO (dimethylsulfoxied)), 20 μL was spread onto sterilized pre-made (yeast extract mannitol and sucrose/yeast extract agar media) plates to reach a final concentrations of 0.5 μg Nile red/mL medium. After inoculation, the plates were incubated overnight at 30°C subsequently; the agar plates were exposed to ultraviolet light (312 nm) after appropriate cultivation periods to detect the accumulation of PHB (Spiekermann *et al.*, 1999). The lighted plates were recorded positives, after that these isolates were selected as positive producers for the subsequent experiments. Fifteen bacterial colonies were isolated,

purified and preserved using enrichment and nutrient agar medium. The efficient isolates were selected according to high production of PHB (poly-β-hydroxybutyrate) were selected and identified on the basis of their morphological, cultural, physiological and biochemical characteristics.

**Identification:** The efficient selected PHB producing bacterial isolates were identified depending morphological, physiological characteristics as described by John (1984) and 16S rDNA as follow:

**Genomic DNA extraction from bacteria:** DNA extraction was carried out following the CTAB method according to Azadeh and Meon (2009).

**Primer design and PCR amplification:** Oligonucleotide primer for 16S rDNA gene was 16S-1f (5'-GCTAGTTGGTGGGGTAA-3', 17 mer) and 16S-2r (5'-GCCATCTCAGTTCGGATTG-3'; 18 mer) were designed on the basis of the sequence of *E. coli* 16S gene (corresponding to positions 247 to 263 and 1291 to 1309; *E. coli* numbering system). Amplification reaction for *Pseudomonas stutzeri* E114 and *Rhizobium elti* E1 was per formed according to (Wilems and Collins, 1993).

**Nucleotide sequence analysis:** The PCR products was purified using a commercial kit (QIA Quick PCR purification kit (Qiagen, Valencia, CA, USA), according to manufacturer's instruction. After purification, the PCR products were sent for sequencing services at Sigma Co. Germany. The 16S gene sequences were aligned using BioEdit software versions 7.0.8 (<http://www.mbio.ncsu.edu/bioEdit>) and searched for sequence similarity to other sequences which are available in the NCBI database at <http://www.ncbi.nih.gov> using Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments were performed on the selected closely related sequence accessions available using bioedit software ([http:// bioedit.edu/](http://bioedit.edu/)).

**Phylogenetic analysis:** Phylogenetic analysis was done based on the nucleotides sequences of 16S gene using mega 4 or tree view software provided by the Biology Workbench Program (<http://workbench.sdsc.edu/>). Number of Data base JYZVMFRR015, K12FNS3P01S.

**PHB production and extraction:** PHB was extracted from paste cells, precipitated and determined as dry weight (g/L) according to the method recommended by Williamson and Wilkinson (1958) and Hahn (1994). Cells were collected by centrifugation at 10,000 rpm from 1 to 5 mL samples of the culture suspended in 10 mL of sodium hypochlorite reagent (pH 10.0-10.5 NaOCl content 5.25-5.5%) according to (Williamson and Wilkinson, 1958). After 1 h at 37°C the reaction

mixture was centrifuged at 5000 rpm for 10 min and the solid pellet was washed successively with 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 for 2 h at 105°C until constant weight and weight was measured. 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.

**Estimation of cell dry weight:** After centrifugation of the culture medium, the supernatant was discarded and the pellet was washed with distilled water. The washed pellet was re-suspended in 1 mL distilled water, transferred to pre-weighed plates and dried to constant weight at 60°C.

**Determination of PHB:** Determination of the amount of PHB was performed chemically. Bacteria were grown on YEM broth and whey broth at 30°C for 48 h on a shaker and without shaker. A sample of culture solution (0.1-0.2 mL) was added to 4 mL of the sample were centrifuged for 30 min to precipitate solution and then left for 1 day. (0.4 mL) of the sample was centrifuged for 30 min to precipitate the PHB. The solid pellet was re-suspended and washed with 1 mL portions of water Acetone and ether. Chloroform was added and allowed to boil in a water bath at 100°C. The settled material after evaporation of chloroform was dried at 40°C for 30 min. The white powder was dissolved in concentrated H<sub>2</sub>SO<sub>4</sub> (2.5 mL) and heated for 10 min at 100°C. After cooling, the solution was measured photometrically at 235 nm against H<sub>2</sub>SO<sub>4</sub> blank (Slepecky and Law, 1960b; Kuniko *et al.*, 1988).

**PHB spectrophotometer assay:** For the spectrophotometer assay of polymer cells were collected by centrifugation at 10000 rpm then the PHB extracted by three previous methods, a sample containing polymer in chloroform is transferred to a clean test tube the chloroform is evaporated and 10 mL of concentrated H<sub>2</sub>SO<sub>4</sub> are 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 concentrations according to Slepecky and Law (1960b) and Kuniko *et al.* (1988).

#### **Factors affecting accumulation of PHB:**

**Cell cultivation:** Conical flask (500 mL) containing 100 ml of each previous medium (yeast extract mannitol for *R. elti* E1 and Sucrose/yeast extract for *P. stutzeri* E114) were sterilized at 121°C for 20 min

and inoculated with 1 mL of inoculum containing 10<sup>7</sup> cfu/mL (yeast extract mannitol or sucrose/yeast extract medium, 10<sup>7</sup> cfu/mL, incubated at 30°C and 150 rpm for 3 days) from the both strains. The inoculated flasks were incubated at 30°C and 150 rpm for 48 h for the both strains. PHB was determined by the described method to select optimum carbon, nitrogen source and C: N ratio for PHB production (Khanna and Srivastava, 2005). Three replicates from each treatment were used.

#### **OPTIMIZATION OF CULTURE MEDIUM AND CONDITIONS**

**pH and temperature optimization:** To determine the optimum pH, experiments were carried out at pH (6, 7 and 8). Cultures were incubated on a rotary shaker at 30°C and 150 rpm for 7 days. To determine the effect of temperature, yeast extract mannitol or sucrose/yeast extract medium with pH of 7 was incubated at 25, 30, 35, 40 and 45°C under 150 r/min.

**Optimization of incubation time:** To determine the effect of incubation period, the inoculated yeast extract mannitol or sucrose/yeast extract medium were incubated at 30°C and pH7 at 150 rpm under different incubation period (24, 48 and 72 h, respectively).

**Optimization of carbon and nitrogen sources for PHB production:** To detect the effect of carbon sources, yeast extract mannitol or yeast extract mannitol or sucrose/yeast extract 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, mannitol, glucose, xylose, lactose, starch, industrial dairy products (like whey) and agricultural residues such as molasses, sugar cane 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).

Conical flask (500 mL) containing 100 mL of medium were sterilized at 121°C for 20 min and inoculated with 1 mL of inoculum containing 10<sup>7</sup> cfu/mL from the both strains. The inoculated flasks were incubated at 30°C and 150 rpm for 48 h. PHB was determined by the described method to select optimum carbon source. Three replicates from each treatment were used.

To detect the effect of nitrogen sources, yeast extract mannitol or sucrose/yeast extract medium was prepared with the optimum carbon source and supplemented with different concentrations of nitrogen (0.5 and 1 g/L) at pH 7 and 30°C. The nitrogen sources included ammonium sulphate (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, ammonium chloride (NH<sub>4</sub>Cl), ammonium nitrate, yeast extract and proteose peptone.

**The best carbon source and different concentrations of the best N source viz., 05 and 1 g/L:** 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 h, PHB yields were extraction and quantified as mentioned before. Three replicates from each treatment were used.

## RESULTS AND DISCUSSION

**Isolation and screening of PHB producing bacteria from different microbial sources:** The present study was carried out to isolate several bacterial isolates from different sources, soil (clay) and root nodules which collected from different localities in Elbehira governorate, Egypt. Fifty five 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 yeast extract mannitol agar medium for root nodule bacteria and sucrose/yeast extract agar medium for soil bacteria and the ability to synthesize PHB granules was confirmed using Nile red staining of PHB granules in the intracellular environment of the isolated bacteria. It was observed that 15 isolates were found to accumulate PHB, one isolate was belong nitrogen bacteria and 14 isolates were belong soil bacteria. All the bacterial isolates were subjected for visual screening for PHB production using Nile-red.

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 (Fig. 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 *et al.*, 1996; Spiekermann *et al.*, 1999).

All isolates subjected for screening by Nile-red staining approach, also quantitative with spectrophotometric assay using H<sub>2</sub>SO<sub>4</sub> 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.*,



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)

Table 1: PHB production by the bacterial isolates

Isolates	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)
E1	2.6±0.08	1.42±0.04	54.60±0.20
E111	1.2±0.12	0.30±0.11	25.00±0.13
E112	0.8±0.11	0.10±0.11	12.50±0.11
E113	0.6±0.14	0.20±0.09	33.30±0.16
E114	0.5±0.11	0.10±0.07	20.00±0.12
E115	1.8±0.12	0.20±0.10	11.10±0.11
E116	2.1±0.10	0.40±0.10	19.04±0.14
E117	2.4±0.16	0.50±0.10	20.80±0.15
E118	1.8±0.15	0.30±0.10	16.70±0.12
E119	1.4±0.09	0.20±0.10	14.30±0.11
E110	1.1±0.13	0.10±0.00	9.10±0.12
E111	0.9±0.12	0.10±0.12	11.10±0.15
E112	1.3±0.13	0.20±0.15	15.40±0.10
E113	1.7±0.17	0.30±0.13	17.50±0.11
E114	5.0±0.06	3.00±0.03	60.00±0.17

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 0.1-3 g/L and the percentage of PHB in these cells was between 9.1 and 60.0% of dry cell weight. While the PHB productivity percentage in *Rhizobium* spp. (E1) was (54.6%). The highest PHB productivity was in soil bacteria designated as E114, the lowest PHB productivity was found in soil bacteria designated E110 (9.1%). It was found that a significant relationship existed between dry cell weight and PHB production.

Out of 15 positive isolates, E1 and E114 were finally selected as promising isolates in PHB production for further studies. 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

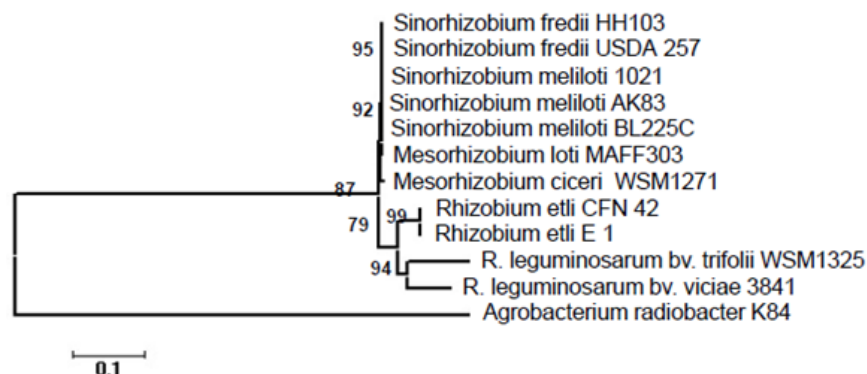


Fig. 2: Dendrogram illustrating the genomic relationship among ten isolates belonging to genus *Rhizobium* revealed by UPGMA cluster analysis. The label at the internal nodes shows the distance and the bar 0.1 represents substitution

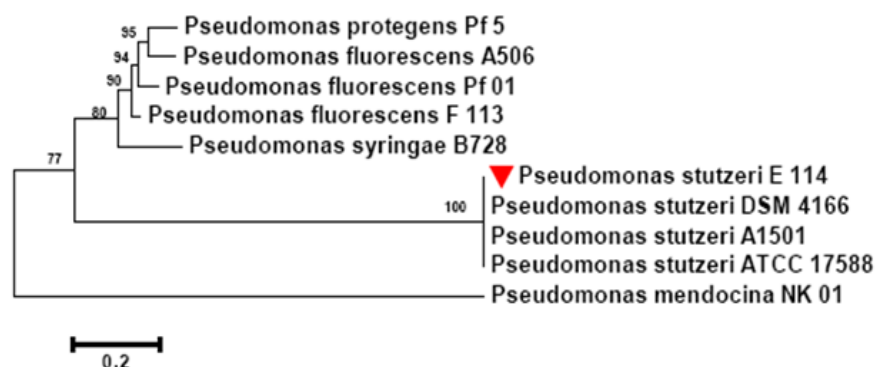


Fig. 3: Dendrogram illustrating the genomic relationship among ten isolates belonging to genus *Pseudomonas* revealed by UPGMA cluster analysis. The label at the internal nodes shows the distance and the bar 0.2 represents substitution

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* (Smet *et al.*, 1983; Suzuki *et al.*, 1986; Taylor and Anthony, 1976; Aremu *et al.*, 2010).

These bacterial strains E1 and E114 were identified according to morphological, physiological as well as using analysis of 16S rDNA (Fig. 2 and 3).

The bacterial strain (E1) was found to be motile, Gram negative, short rods. The colonies on Yeast Extract Mannitol Agar (YEMA) were circular, non-spreading, non-chromogenic, easily emulsifiable in water and odourless. Streaking of the isolates on YEMA containing congo red or Bromothymol Blue (BTB) gave normal reactions. Fast growing rhizobia such as *Rhizobium* sp., have an acid reaction, turning the medium yellow and also it's not absorb Congo red when plates are incubated in the dark. The formed zone in litmus milk gave alkaline reaction. The growths of these isolates on yeast extract mannitol broth medium showed uniform turbidity.

The bacterial strain (E114) was gram-negative, motile, rods and oxidase positive. According to the 16S rDNA analysis, the phylogenetic tree of the isolated bacteria (E1) and (E114) and related bacterial species based on the 16S rDNA sequence is provided in Fig. 2 and 3, respectively. It can be clearly seen that the isolated bacterial strains was included in the genus *Rhizobium* (Fig. 2) and *Pseudomonas* (Fig. 3) and closely related to the species *Rhizobium elti* E1 and *Pseudomonas stutzeri* E114. It showed the highest sequence similarities with *Rhizobium elti* CFN 42 (99%) (Fig. 2) and *Pseudomonas stutzeri* DSM 4166 (100%) Fig. 3 for *Rhizobium elti* E1 and *Pseudomonas stutzeri* E114, respectively.

**Optimization of cultural conditions for PHB production:** Natural polymers are synthesized and accumulated by a diverse group of microorganisms. Hydroalkanoic acids have been detected in some microorganisms and if they are exploited for optimized PHB production this may reduce production cost and help in large scale uses. Keeping this in mind, in this study we optimized the conditions for PHB production by the isolated microbe of *R. elti* E1 and *P. stutzeri* E114.

Table 2: Effect of incubation time on PHB production by production by *R. elti* E1 and *P. stutzeri* E114

Strains	Incubation time (h)	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)
<i>R. elti</i> E1	24	2.90±0.23	1.5±0.30	51.7±0.50
	48	4.90±0.20	3.4±0.11	69.4±0.10
	72	4.50±0.35	2.2±0.23	48.9±0.33
<i>P. stutzeri</i> E114	24	3.01±0.10	1.6±0.10	53.2±0.10
	48	5.30±0.12	3.7±0.09	69.8±0.12
	72	4.40±0.21	2.1±0.31	47.7±0.42

Table 3: Effect of pH on PHB production by production by *R. elti* E1 and *P. stutzeri* E114

Strains	pH	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)
<i>R. elti</i> E1	6	1.6±0.18	0.7±0.19	37.5±1.10
	7	5.0±0.17	3.2±0.15	64.0±0.26
	8	1.2±0.17	0.4±0.18	33.3±2.10
<i>P. stutzeri</i> E114	6	1.9±0.15	0.8±0.16	42.1±2.20
	7	5.5±0.13	3.6±0.11	65.5±0.13
	8	1.4±0.19	0.5±0.21	35.7±1.30

Table 4: Effect of temperature on PHB production by production by *R. elti* E1 and *P. stutzeri* E114

Strains	Temperature	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)
<i>R. elti</i> E1	20	1.1±0.20	0.3±0.15	27.3±3.1
	30	5.2±0.18	3.4±0.16	65.4±2.1
	40	0.0±0.00	0.0±0.00	0.0±0.0
<i>P. stutzeri</i> E114	20	1.2±0.19	0.4±0.15	33.3±2.7
	30	4.9±0.16	3.2±0.12	63.3±1.1
	40	0.0±0.00	0.0±0.00	0.0±0.0

Table 2 shows the effect of incubation time on PHB production by *R. elti* E1 and *P. stutzeri* E114. The PHB yields increased with time dependent manner and highest yield 3.4 and 3.7 g/L was obtained after 48 h for growth of *R. elti* E1 and *P. stutzeri* E114, respectively. The PHB yield decreased to 2.1 and 2.2 g/L at 72 h by *R. elti* E1 and *P. stutzeri* E114, respectively.

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.*, 1986; 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.

**Effect of different pH values on PHB yield:** Data presented in Table 3 showed that, out of the different pHs of media tested, pH 7.0 was found to be optimum for maximum PHB production by *R. elti* E1 and *P. stutzeri* E114. The highest PHB production of 3.2 and 3.7 g/L culture was produced by *R. elti* E1 and *P. stutzeri* E114 at pH 7.0. All the strains were found to produce lower yields showing at pH 6 and 8 and pH 7.0 was found as the best pH for the both strains. 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% 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.

Data presented in Table 4 showed that, incubation at 30°C was found to be optimum and has produced 3.4 and 3.2 g/L after 48 h of incubation under stationary conditions for growth of *R. elti* E1 and *P. stutzeri* E114 at pH 7. The highest PHB production of 3.2 and 3.7 g/L culture was produced by *R. elti* E1 and *P. stutzeri* E114 at 30°C. All the strains were found to produce lower yields showing at 20 and 40°C. As shown in Table 3 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 4).

Table 5: Effect of different carbon source on PHB production by *R. elti* E1 and *P. stutzeri* E114

Strains	Carbon source	1%			2%			
		Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)	
<i>R. elti</i> E1	Sucrose	2.90±0.100	1.00±0.140	37.9±2.10	3.60±0.200	1.90±0.200	52.8±2.40	
	Mannitol	3.20±0.070	1.40±0.090	43.8±1.10	4.80±0.110	2.90±0.130	60.4±1.60	
	Glucose	1.20±0.110	0.20±0.090	16.7±1.30	1.40±0.300	0.40±0.080	28.6±2.10	
	Xylose	0.80±0.080	0.14±0.050	17.5±2.10	0.90±0.050	0.20±0.060	22.2±1.70	
	Lactose	0.90±0.010	0.20±0.010	22.2±1.10	1.10±0.010	0.30±0.001	27.3±1.10	
	Molasses	2.20±0.100	0.70±0.100	31.8±0.10	3.50±0.100	1.20±0.100	37.1±0.10	
	Starch	0.05±0.010	0.01±0.001	10.0±0.60	0.06±0.001	0.01±0.001	16.7±1.10	
	Whey	4.00±0.110	0.70±0.030	17.5±1.20	4.70±0.300	0.20±0.070	25.5±1.30	
	Sugar cane bagasse	0.60±0.030	0.08±0.020	13.3±1.70	0.70±0.070	0.10±0.005	14.3±1.70	
	Rice straw hydrolysate	0.50±0.110	0.07±0.010	14.0±1.11	0.60±0.110	0.10±0.009	16.7±1.11	
	<i>P. stutzeri</i> E114	Sucrose	4.00±0.100	2.20±0.090	55.0±1.20	5.50±0.300	3.40±0.110	61.8±0.40
		Mannitol	2.80±0.400	1.20±0.300	42.9±2.30	3.60±0.900	1.80±0.700	50.0±1.50
Glucose		1.40±0.110	0.40±0.070	28.6±1.50	1.80±0.070	0.70±0.050	38.9±1.11	
Xylose		1.10±0.100	0.20±0.002	18.2±1.10	1.40±0.200	0.40±0.001	28.6±1.30	
Lactose		1.20±0.010	0.40±0.001	33.3±1.11	1.50±0.005	0.60±0.001	40.0±1.10	
Molasses		2.50±0.030	1.10±0.030	44.0±1.30	4.20±0.003	2.20±0.001	52.4±2.10	
Starch		1.10±0.010	0.20±0.001	18.2±1.02	1.60±0.002	0.40±0.001	25.0±1.01	
Whey		4.50±0.300	0.90±0.010	20.0±0.80	5.00±0.200	1.40±0.030	28.0±0.90	
Sugar cane bagasse		0.90±0.002	0.15±0.001	16.7±1.01	1.10±0.001	0.24±0.001	21.8±1.01	
Rice straw hydrolysate		0.58±0.011	0.08±0.001	13.8±1.01	0.67±0.001	0.10±0.001	14.9±1.10	

**Effect of different carbon sources on PHB production by the selected isolates of bacteria:** The data illustrated in (Table 5) 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, *R. elti* E1 and *P. stutzeri* E114 had shown preference for carbon sources like, mannitol, sucrose, glucose, xylose, lactose, molasses, starch, sugar cane bagasse and rice straw hydrolysate.

The results interpreted significant differences for *R. elti* E1 and *P. stutzeri* E114, carbon sources and their interactions as well. In general, there was an increasing of PHB production with an increase in carbon sources concentration from 1 to 2% by the both strains. Among the strains, *P. stutzeri* E114 was found to be a significantly higher PHB producer compared to *R. elti* E1.

The highest PHB productivity was observed by *R. elti* E1 growing on mannitol being 1.4-2.9 (43.8-60.4) and is dependent on mannitol concentration, while they were 2.2-3.4 (55-61.8%) by *P. stutzeri* E114 on sucrose medium.

Among the strains, *P. stutzeri* E114 was found to be a significantly higher PHB producer compared to *R. elti* E1. It produced a mean PHB in range from 0.80-3.4 g/L. *R. elti* E1 utilize carbon sources and produced PHB in range from 0.07 to 2.9 g/L. Among different N sources, ammonium sulphate was found to be the best N source. The next promising C sources were glucose, xylose and lactose and followed by the raw materials such as starch, whey, sugar cane bagasse and rice straw hydrolysate.

Among the different carbon sources tested to evaluate their effects on PHB yield, mannitol and sucrose were found to be the best carbon source for *R. elti* E1 and *P. stutzeri* E114, respectively. The lowest

PHB production was obtained with rice straw hydrolysate on dry cell weight basis of *R. elti* E1 and *P. stutzeri* E114, respectively.

Mannitol yielded a mean PHB of 2.9 g/L by *R. elti* E1 more than sucrose. On the other hand, sucrose yielded a mean PHB of 2.9 g/L by *P. stutzeri* E114 more than mannitol. This was followed by all the carbon sources. It was shown that the highest concentration from raw materials enhanced PHB synthesis in *R. elti* E1 and *P. stutzeri* E114.

Poly-β-Hydroxybutyric acid (PHB) was produced from glucose (ranged from 0.2 to 1.8 g/L), xylose (ranged from 0.14 to 0.4 g/L), lactose (ranged from 0.2 to 0.6 g/L), molasses (ranged from 0.7 to 2.2 g/L), starch (ranged from 0.01 to 0.4), sugar cane bagasse (ranged from 0.08 to 0.24) and rice straw hydrolysate (ranged from 0.07 to 0.1 g/L) by using *R. elti* E1 and *P. stutzeri* E114, 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. *R. elti* E1 and *P. stutzeri* E114 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

Table 6: Effect of different nitrogen source on PHB production by *R. elti* E1 and *P. stutzeri* E114

Strains	Carbon source	0.5 g/L			1 g/L		
		Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)	Cell dry weight (g/L)	PHB (g/L)	PHB yield (%)
<i>R. elti</i> E1	Ammonium sulphate	3.4±0.08	2.1±0.04	61.8±0.80	4.4±0.11	3.1±0.09	70.5±1.10
	Ammonium chloride	2.9±0.30	1.2±0.15	41.4±2.10	3.2±0.25	1.6±0.13	50.0±2.10
	Ammonium nitrate	2.4±0.20	0.9±0.09	37.5±1.20	2.7±0.09	1.1±0.10	40.7±3.10
	Yeast extract	1.8±0.11	0.4±0.09	22.2±1.30	2.2±0.11	0.6±0.03	27.3±1.11
	Proteose peptone	2.1±0.00	0.2	9.5	2.6	0.4	15.4
<i>P. stutzeri</i> E114	Ammonium sulphate	4.1±0.10	2.8±0.03	68.3±1.03	5.2±0.03	3.9±0.07	75.0±0.50
	Ammonium chloride	3.1±0.20	1.3±0.20	41.9±1.80	3.6±0.20	1.9±0.25	52.8±1.30
	Ammonium nitrate	2.7±0.13	1.1±0.21	40.7±2.10	3.1±0.40	1.5±0.30	48.4±1.70
	Yeast extract	2.1±0.14	0.5±0.11	23.8±1.11	2.5±0.30	0.9±0.11	36.0±1.30
	Proteose peptone	2.6±0.30	0.4±0.13	15.4±1.05	3.1±0.30	0.7±0.13	22.6±2.01

from hemicellulosic hydrolysates and cheese whey by *P. cepacia* may be possible (Young *et al.*, 1994).

Whey permeate 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 ( $\beta$ -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 (Fu and Tseng, 1990). Gerhart (2008) reported PHA production from whey permeate using *Pseudomonas hydrogenovora*. Yellore and Desai (1998) and Nath *et al.* (2005) reported production of Poly ( $\beta$ -Hydroxybutyrate) (PHB) from disaccharides like lactose and sucrose by *Methylobacterium* sp., ZP 24 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). 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). In confirmation of these results, Page (1992a) reported that the strains of *Rhizobium* produced less PHB in Yeast Extract Mannitol (YEM) broth media with different carbon (glucose, sucrose and arabinose).

**Effect of different nitrogen sources on PHB production by *R. elti* E1 and *P. stutzeri* E114:** Data

pertaining to PHB yields produced by *R. elti* E1 and *P. stutzeri* E114 in presence of different concentrations from N sources and the best carbon source (mannitol and sucrose 2%) are presented in Table 6. There were significant differences for the isolates, N sources and their interactions. 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 1 g/L by *R. elti* E1 and *P. stutzeri* E114.

Among the strains, *P. stutzeri* E114 was found to be a significantly higher PHB producer compared to *R. elti* E1. It produced a mean PHB in range from 0.4-3.9 g/L. *R. elti* E1 utilize nitrogen sources and produced PHB in range from 0.2 to 3.1. Among different N sources, ammonium sulphate was found to be the best N source. It produced a mean PHB of 2.1-3.1 and 2.8-3.9 g/L by *R. elti* E1 and *P. stutzeri* E114, respectively. The next promising N sources were ammonium chloride with (1.2 -1.9 g/L), ammonium nitrate with (0.9-1.5 g/L), yeast extract (0.4-0.9 g/L), proteose peptone (0.2-0.7 g/L) PHB yields. The highest PHB was obtained in ammonium sulphate followed by ammonium chloride.

Proteome 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) and 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 (the best nitrogen source) on PHB production by *R. elti* E1 and *P. stutzeri* E114:** PHB yields produced by *R. elti* E1 and *P. stutzeri* E114 when grown on different concentrations of the best N source (ammonium sulphate) in presence of the best carbon source (mannitol and sucrose 2%) are presented in



Table 7: Influence of different concentrations of ammonium sulphate on PHB production by *R. elti* E1 and *P. stutzeri* E114

Strains	Carbon source	0.5 g/L			1 g/L			1.5 g/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 (%)
<i>R. elti</i> E1	Ammonium sulphate	3.3±0.3	1.9±0.4	57.6±3.1	4.7±0.03	3.6±0.09	76.6±0.8	4.1±0.02	2.9±0.2	70.7±0.10
<i>P. stutzeri</i> E114	Ammonium sulphate	3.7±0.2	2.3±0.3	62.2±4.2	5.3±0.04	4.1±0.07	77.4±0.5	4.0±0.03	2.8±0.3	70±0.14

Table 8: Effect of different C: N ratios of medium on PHB yields by *R. elti* E1 and *P. stutzeri* E114

Strains	Carbon source	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 (%)
<i>R. elti</i> E1	Ammonium sulphate	3.7±0.11	2.3±0.12	62.2±2.1	4.4±0.04	3.6±0.08	81.80±0.2	4.9±0.3	3.2±0.2	65.3±2.1
<i>P. stutzeri</i> E114	Ammonium sulphate	3.9±0.11	2.5±0.13	64.1±2.3	5.3±0.07	4.4±0.09	83.02±0.3	5.2±0.4	3.6±0.3	69.2±1.9

Table 7. *R. elti* E1 and *P. stutzeri* E114 produced a mean PHB yield of 3.6 and 4.1 g/L, respectively. Out of three concentrations of ammonium sulphate, it was found that of ammonium sulphate, at 1.0 g/L supported the highest PHB production (0.377 g/100 mL) when compared to other levels. In general, there was an increasing of PHB production with an increase in ammonium sulphate concentration from 0.5 to 1.0 g/L. Amongst the interaction, it was found that *P. stutzeri* E114 were found to produce the highest PHB yield of 4.1 at 1.0 g/L concentration which was significantly higher than the PHB produced by *R. elti* E1 (3.6 g/L). In fact, *P. stutzeri* E114 was the highest PHB producer at all levels of ammonium sulphate. The presence of higher concentration of nitrogen in the culture medium was inhibitor for the accumulation of PHB. Hence, the concentration of the best nitrogen source needs to be optimized. Different concentrations of ammonium sulphate were amended to the medium containing mannitol or sucrose (2%) as the best carbon source. From the studies, it was revealed that ammonium sulphate at 1.0 g/L was the optimum level for *R. elti* E1 and *P. stutzeri* E114. When ammonium sulphate concentration was increased from 0.50 to 1.00 g/L, the PHB accumulation was also increased. But, at 1.5 g/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 1 g/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 by *R. elti* E1 and *P. stutzeri* E114:** Different C:N ratios were maintained using the best carbon and nitrogen sources in yeast extract mannitol and sucrose/yeast extract as well as their effects on PHB production were studied. The data are presented in Table 8 showed that, *R. elti* E1 and *P. stutzeri* E114 were found to be significantly higher PHB producer mean where the PHB of 4.4 and 5.5 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. Interaction of *P. stutzeri* E114 with different C: N ratio were found to be significantly higher than *R. elti* E1. The highest yield of PHB (5.5 g/L culture) was by *P. stutzeri* E114 at 20:1. This was followed by *R. elti* E1 with 4.4 g/L. *P. stutzeri* E114 was found to produce the highest PHB content at 25:1 C:N ratio which was 4.3 g/L culture.

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. To exploit this, the C:N ratios were adjusted to 15:1, 20:1 and 25:1 using mannitol or sucrose and ammonium sulphate as C and N sources for *R. elti* E1 and *P. stutzeri* E114, respectively. The PHB yield analysis showed that there was the highest accumulation of PHB at 20:1 C: N ratio by *R. elti* E1 and *P. stutzeri* E114, respectively. *P. stutzeri* E114 was efficient to produce the PHB yield of 4.4 g/L culture which is significantly higher than the PHB produced by *R. elti* E1 (3.6 g/L culture). At 25:1 C: N ratio, PHB yields were significantly lower by *R. elti* E1 and *P. stutzeri* E114 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. This was probably due to the substrate inhibition. Similar observations were made by other scientists. Belfaas *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 (1996) on PHB production have been reported.

## CONCLUSION

It can be conclude that optimum culture conditions for PHB production by *R. elti* E1 and *P. stutzeri* E114. The existence of carbon source where the sugar of mannitol and sucrose were considered the best carbon source to produce PHB compared to the other carbon sources by *R. elti* E1 and *P. stutzeri* E114, respectively. 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 the both strains. 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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