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Full Length Research Paper

Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria

Paramjit Singh and Nitika Parmar*

Biology Program, California State University Channel Islands, 1 University Drive, Camarillo, California, 93012, USA.

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Bioplastics are naturally occurring biodegradable polymers made from polyhydroxyalkanoates (PHA) of which poly 3-hydroxy butyric acid (PHB) is the most common. PHB serves as an energy storage molecule and accumulates intracellularly as storage granules in microbes. This work utilized the biodiversity of bacteria to isolate various species from different environments and screen them for their ability to produce PHB. Sixteen unknown samples were collected, assayed and compared with known bioplastic producers (*Ralstonia, Bacillus and Pseudomonas*). These species were identified by 16S rRNA sequencing. Conditions were extensively optimized by varying the temperature, carbon, nitrogen and substrate sources for maximal PHB production. Presence of accumulation of PHB in these strains was confirmed by microscopic staining. This is the first report to experimentally demonstrate the synthesis of PHB by two novel species, *Rahnella aquatilis* and *Stenotrophomonas maltophilia*. *R. aquatilis* was collected from a children's plastic toy, while *S. maltophilia* was collected from the sand of a local beach. The phenotypic profiles of these two species were subsequently studied using phenotype microarray panels which allowed the testing of the effect of more than 90 different carbon, nitrogen, sulfur and phosphorus sources as well as pH on the growth characteristics of these strains.

Key words: Poly 3-hydroxy butyric acid (PHB), polyhydroxybutyrate, bioplastics, phenotype microarray, *Rahnella*, *Stenotrophomonas.*

INTRODUCTION

Bioplastic is defined as a form of plastic synthesized from renewable resources such as plant starch and microbial species. Bioplastics are made from a compound called polyhydroxyalkanoate (PHA). The family of PHAs includes several polymeric esters such polyhydroxybutyrates, polyhydroxybutyrate co-hydroxyvalerates (PHBV), polyhydroxybutyrate co-hydroxyhexanoate (PHBHx) and polyhydroxybutyrate co-hydroxyoctonoate (PHBO). Poly 3-hydroxybutyric acid (PHB) is the most common natural microbial PHA. In some microbial species, accumulation of PHA occurs during the presence of excess carbon and a limitation of nitrogen sources (Verlinden et al., 2007). PHAs produced in response to stressful conditions serve as energy storage molecules to be utilized when common energy sources are absent (Solaiman and Ashby, 2005). The plastic polymers accumulate intracellularly as light-refracting amorphous storage granules in these organisms (Barnard and Sander, 1989). PHB is synthesized from acetyl-CoA using three enzymatic steps (Krans et al., 1997). From a biotechnological point of view, the ability of bioplastics to be biodegradable makes them a desirable substitute for petrochemical-based plastic, an environmental pollutant (Lee, 1996). Increased production of bioplastics can significantly reduce carbon dioxide emissions, curtail plastic waste generation and decrease consumption of fossil fuels. Although, the high cost of industrial production and recovery of bioplastics is presently not able to compete with traditional ways of synthetic plastic production, fermentation processes have

*Corresponding author. E-mail: nitika.parmar@csuci.edu. Tel: 805-437-8873. Fax: 805-437-8895.

Abbreviations: PHB, Polyhydroxybutyrate; **PBS,** phosphate buffered saline; **PM,** phenotype microarray; **PHA,** polyhydroxyalkanoate; **PHBV,** polyhydroxybutyrate co-hydroxyvalerates; **PHBHx,** polyhydroxybutyrate co-hydroxyhexanoate; **PHBO,** polyhydroxybutyrate co-hydroxyoctonoate; **ATCC,** American type culture collection; **PBS,** phosphate buffered saline; **PCR,** polymerase chain reaction; **BLAST,** Basic Local Alignment Search Tool.

Table 1. Sites from where the unknown bacteria were collected.

Unknown	Site of collection
1	Outdoor plastic chair 1
2	Outdoor plastic chair 2
3	Trash bag
4	Trash can
5	Refrigerator liner
6	Kids' toys
7	Sprinkler 1
8	Sprinkler 2
9	Wooden bench
10	Kayak at Channel Island Harbor
11	Dock of Channel Island Harbor
12	Ventura beach marsh
13	Ventura beach sand
14	Piece of plastic, Malibu beach
15	Backyard soil
16	Malibu beach soil

been greatly improved by the use of recombinant organisms capable of producing these polymers at a high rate, utilizing cheap carbon sources such as molasses (Luengo et al., 2003). It is estimated that bioplastics will capture 30% of the total plastics market within the next decade.

Although, a number of bacterial species have been identified which produce PHB, the potential to discover and identify novel species with vastly superior production capacity remains untapped. Isolation, identification and genetic manipulation of other microbes which produce many bioplastics with different structures, properties and applications indicate a promising future for the industrialization of bioplastics. A critical component for the commercialization of bioplastic production is the optimization of conditions for maximal synthesis of PHB by varying culture parameters such as temperature, pH and type of carbon source.

PHB has been shown to be naturally produced by several bacteria such as *Alcaligenes, Pseudomonas, Bacillus, Rhodococcus, Staphylococcus* and *Micrococcus* (Anderson and Dawes, 1990; Verlinden et al., 2007). This study focuses on the isolation and identification of novel species of bacteria capable of producing PHB. Bacteria from diverse environments were screened and their PHB production capability was determined under different growth and nutritional conditions. The known PHB producing species- *Pseudomonas olevorans* (Huisman et al., 1989), *Ralstonia eutropha* (Anderson and Dawes, 1990; Steinbuchel, 1991), and *Bacillus megaterium* (Kulpreecha et al., 2009) were utilized for determining the optimal conditions for PHB production. Conditions that were varied included type of carbon sources, temperature and types of substrate for PHB production. The unknown bacteria collected were identified by 16S rRNA sequencing and PHB accumulation was confirmed by microscopic staining techniques. The phenotypic profiles of these novel PHB producers were analyzed using phenotype microarray panels, a powerful platform enabling the monitoring of the effects of hundreds of different nutrients on cell growth simultaneously.

MATERIALS AND METHODS

Microbial strains

Three known PHB producing bacteria, *P. oleovorans* (ATCC 8062), *R. eutropha* (ATCC 17699), and *B. megaterium* (ATCC 89) were obtained from American Type Culture Collection (ATCC). DH5- α strain of *Escherichia coli* was used as the negative control. Sixteen unknown microbial strains were screened for PHB production. These strains were collected from diverse environments as indicated in Table 1. The cultures were obtained by using a sterile swab and streaking onto LB plates. Direct swabs were taken from solid objects and liquids. Isolated colonies from these plates were selected and subsequently re-streaked on LB plates. Cultures were maintained on LB agar and minimal media agar plates at 4°C and sub cultured monthly. Glycerol stocks were prepared in LB media supplemented with 20% (w/v) glycerol and stored at -80°C.

Media and culture conditions

All strains were grown either in rich (LB) or synthetic (minimal) liquid media. Minimal media consisted of: M9 Salts (30 g/l $Na_2HPO_4.7H_20$, 15 g/lKH_2PO_4, 2.5 g/l NaCl, 5 g/l or 18.5 mM NH_4Cl), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% (w/v) glucose.

To analyze the effect of different substrates on PHB production, four different compounds were utilized: Ethyl heptanoate (ACROS), ethyl caproate (ACROS), diethyl-L-Malate (Wako) and propionic acid (Fisher). These compounds were added to the minimal media at a final concentration of 0.2% (v/v). To analyze the effect of different carbon sources on PHB production, six different sugars were utilized-D-xylose (Fisher), sorbose (MP Biomedicals), fructose (Acros), glucose (Fisher), lactose (Fisher) and glycerol (Fisher). These sugars were used in the minimal media at a final concentration of 0.4% (w/v). To analyze the effect of nitrogen limitation on PHB production, minimal media (containing glucose) was prepared with varying concentrations of NH_4Cl- 2, 5, 10 20, 50 and 90 mM. In order to analyze the effect of cytidine on PHB production, cytidine (Stock of 5% solution in water) was added to minimal media (containing glucose) instead of NH_4Cl as the nitrogen source at a final concentration of 0.2% (w/v).

All strains were cultured in 3ml of appropriate media in the presence or absence of substrate. Cultures were seeded at 0.02% cell density at day 0. For growth assays, cultures were grown either at 30 or 37°C in a shaker for 30 h (LB media) and 4 days (minimal media). Growth was monitored by removing aliquots of the cultures at the indicated time points and measuring the absorbance at 600 nm. For PHB assays, all cultures were grown in minimal media at 30°C for 5 days.

PHB extraction and quantification

PHB extraction was done by using the dispersion method of sodium

hypochlorite and chloroform (Chang et al., 1994; Law and Slepecky, 1961) with minor modifications. Cells were collected by centrifugation at 15,000 rpm for 10 min at room temperature followed by a wash in phosphate buffered saline (PBS). Cell pellets were air-dried for 20 min at room temperature and their weights determined. Chloroform and 6% sodium hypochlorite were added to the cell pellets according to the following ratio- 12.5 ul chloroform to 12.5 ul of 6% (v/v) sodium hypochlorite per mg of pellet weight. Following this addition, the mixture was placed at 30°C overnight. The dispersion was then, centrifuged at 8,000 rpm for 10 min at room temperature resulting in a three phase solution with the bottom phase of chloroform containing the PHB. The chloroform phase solution was carefully transferred to a fresh tube and its volume measured. 5X volumes of a mixture of methanol and water (7:3 v/v) were added to the chloroform solution. The precipitate was recovered by centrifugation at 15,000 rpm for 10 min at room temperature. 0.1 ml of concentrated sulfuric acid was added to the resulting pellet and the contents were transferred to glass tubes. The mixture was then boiled for 20 min at 100°C and cooled. PHB content in this mixture was quantified by measuring the absorbance at 235 nm. PHB concentration was normalized over cell pellet weights. Standard curve for PHB was prepared using poly (3-hydroxybutyric acid) from Sigma as the standard to determine PHB content. Stock solution of the standard was 200 µg of PHB/ml of chloroform.

Microscopic visualization of PHB-producing bacteria

PHB producing bacteria was detected using the lipophilic stain Sudan black (Fisher). Sudan black stain was prepared as a 0.3% solution (w/v) in 60% ethanol. Smears of PHB producing bacteria were prepared on glass slides and heat fixed. Samples were stained for 10 min with Sudan black solution, rinsed with water and counter-stained with 0.5% safranin (BD Sciences) for 5 s. Stained samples were observed under oil immersion at 1000x magnification with direct bright-field illumination using an Olympus CX31 microscope with a Lumenera infinity 2 CCD camera.

rRNA sequencing

16S rRNA sequencing was performed using a commercial custom service, Laragen, Inc. (Los Angeles, CA). Briefly, genomic DNA from the bacterial colony was isolated and the first 500 bp of 16S rDNA were PCR amplified with universal bacterial primers and sequenced. The unknown bacterium was identified using GenBank database.

Phenotype microarray analysis

Phenotype microarray panels were obtained from Biolog, Inc. (Hayward, CA). Four different panels were used: PM1A (Carbon and energy sources), PM3B (Nitrogen sources), PM4A (Phosphorus and sulfur sources) and PM10 (pH). Growth analyses were performed according to protocols provided by the manufacturer. Cells were grown overnight in LB media at 37°C following which the cells were centrifuged at 3000 rpm for 10 min. Pellet was washed with sterile water and cells were then suspended in IF0a- GN/GP base inoculating fluid (Biolog) at a density corresponding to 85% transmittance. The suspensions were then suspended in carbon, nitrogen, sulphur or phosphorous free versions of the medium containing Biolog dye mix A and seeded onto appropriate PM plates at a volume of 100 µl/well. Plates were incubated at 30°C to allow the development of sufficient purple color (~24 h) which was quantified at A570 using a microplate reader (Biotek Instruments, VT).

RESULTS

Effect of temperature on growth of cultures

The aim of the present study was to identify and characterize novel strains of bacteria capable of producing polyhydroxybutyrate. Sixteen microbial samples were collected from diverse areas (Table 1) and numbered 1 through 16. Ideal growth conditions for the unknown cultures were determined by conducting optimization trials on known PHB producers: *P. oleovorans, R. eutropha* and *B. megaterium*; *E. coli* served as a negative control (Dai and Reusch, 2008). Cultures were grown in both rich (LB) and synthetic (minimal) media at 30 and 37°C and growth was monitored spectrophotometrically at A600. As observed in Figure 1, all four strains had similar growth profiles at 30°C (panel A) and 37°C (panel B) in LB media; in minimal media, growth was better at 30°C (Figure 2A and B, respectively). Based on these observations, all subsequent experimentation in this study involving PHB production was conducted at 30°C.

Synthesis of PHB by known bioplastic producers

The PHB producing capacity of these microbes was measured using the chloroform-sodium hypochlorite dispersion method (Chang et al., 1994). A large number of compounds have been shown to serve as substrates for the synthesis of PHBs (Caballero et al., 1995; Huisman et al., 1989; Sankhla et al., 2010; Yao et al., 1999). Four different compounds: caproate, heptanoate, malate and propionate, were tested in this study. The four strains were grown in minimal media supplemented with 0.2% (v/v) of each of these substrates individually for five days at 30°C and PHB produced was quantified. Heptanoate was found to be the best substrate (Figure 3). *P. oleovorans* and *R. eutropha* showed a 10 and 15 fold increase in PHB synthesis in the presence of substrate; no significant amount of PHB was produced by *B. megaterium,* which suggests it may require other specific substrates or alternate energy sources for PHB synthesis.

To determine whether presence of heptanoate itself had any impact on the growth rate of the strains, growth profiles of these species were monitored again in minimal media in the presence of 0.2% heptanoate, both at 30°C (Figure 4A) and 37°C (Figure 4B). Results indicate that, presence of heptanoate in the growth media does not inhibit cell growth (Compare panel A of Figures 2 and 4, cell growth in the absence and presence of heptanoate, respectively) and hence, heptanoate is not likely to compromise the amount of PHB produced due to lower cell density/biomass.

Effect of carbon source on PHB synthesis

PHB production has been previously shown to be

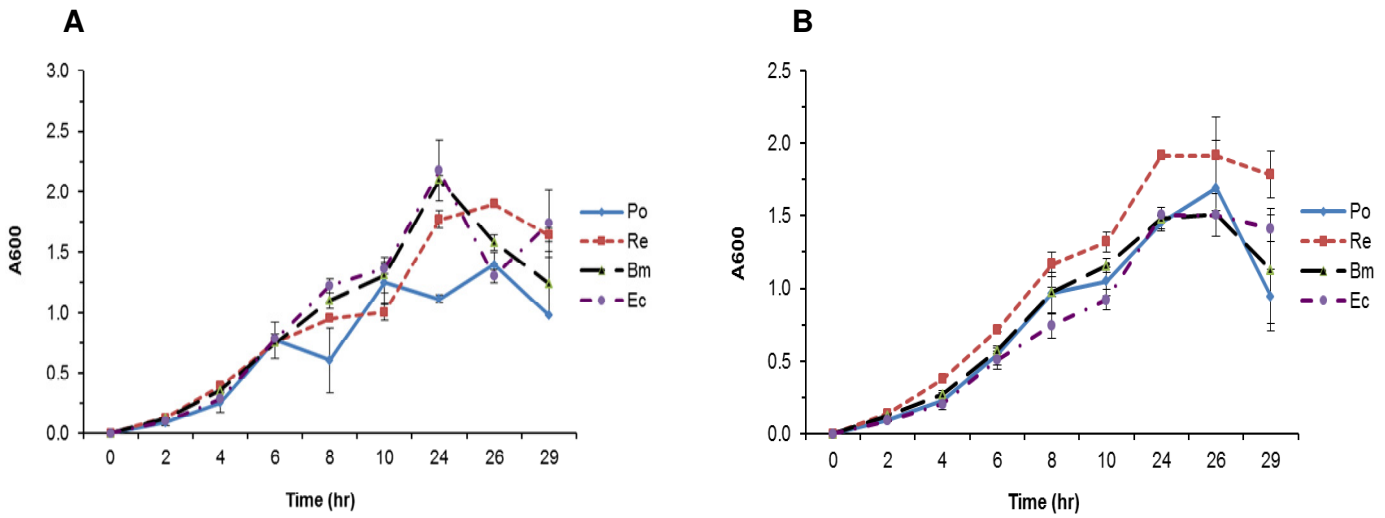

Figure 1. Growth profile of known PHB producing bacteria in rich media. Cultures were grown in LB media at 30°C (panel A) and 37°C (panel B). Growth was monitored by measuring the absorbance at 600 nm as a function of time. Seeding for all cultures at time 0 h was done at an A600 of 0.02. Cultures were grown in triplicates. (*Po- P. oleovorans; Re- R. eutropha; Bm-B. megaterium; Ec- E. coli*.

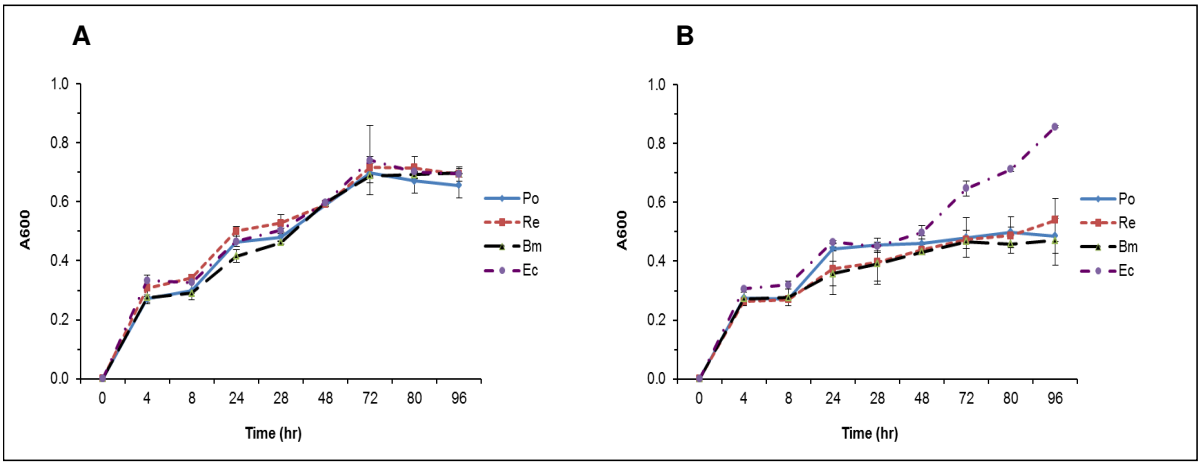

Figure 2. Growth profile of known PHB producing bacteria in synthetic media. Cultures were grown in minimal media at 30°C (panel A) and 37°C (panel B). Growth was monitored by measuring the absorbance at 600 nm as a function of time. Seeding for all cultures at time 0 h was done at an A600 of 0.02. Cultures were grown in triplicates. Po, *P. oleovorans;* Re, *R. eutropha;* Bm, *B. megaterium;* Ec, *E. coli*

affected by the carbon source used (Arun et al., 2009; Khanna and Srivastava, 2005). In this study, six carbon sources were tested. These include glucose, glycerol, lactose, sorbose, fructose and xylose. The known strains were cultured in minimal media containing 0.4% (w/v) of these sugars for five days in the absence and presence of heptanoate (Figure 5, panels A and B, respectively) following which PHB extraction was performed. Although, glucose is the preferred source in the absence of heptanoate, the preference for sugars changes in the presence of heptanoate. The increase in PHB synthesis in media lacking any carbon source but containing heptanoate suggests that, heptanoate could itself serve as a compensatory carbon source besides serving as the provider of the carbon skeleton for PHB synthesis.

Synthesis of PHB by the unknown microbial species

The sixteen unknown cultures were analyzed for PHB synthesis based on conditions optimized for the known cultures. It is important to mention that, although the unknown strains may not be synthesizing PHB specifically, but rather other PHA polymers such as poly (3-hydroxyvalerate) or even copolymers, the assay that is being used in this study has been routinely used for the

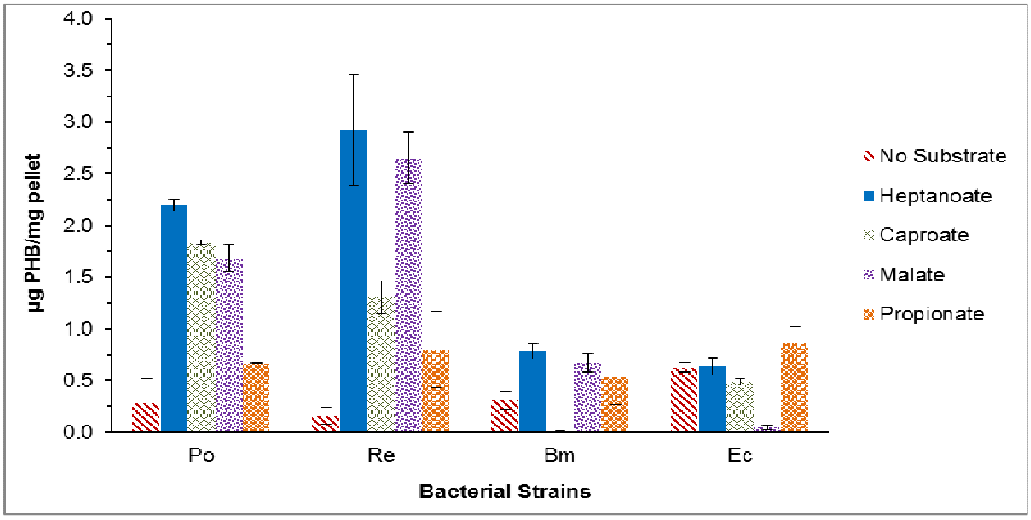

Figure 3. Effect of different substrates on the production of PHB in known PHB producing bacteria. Cultures were grown at 30°C for five days in 3 ml of minimal media lacking or containing four different substrates at a concentration of 0.2% (v/v). Assays were done in triplicates. Po, *P. oleovorans;* Re, *R. eutropha;* Bm, *B. megaterium;* Ec, *E. coli*.

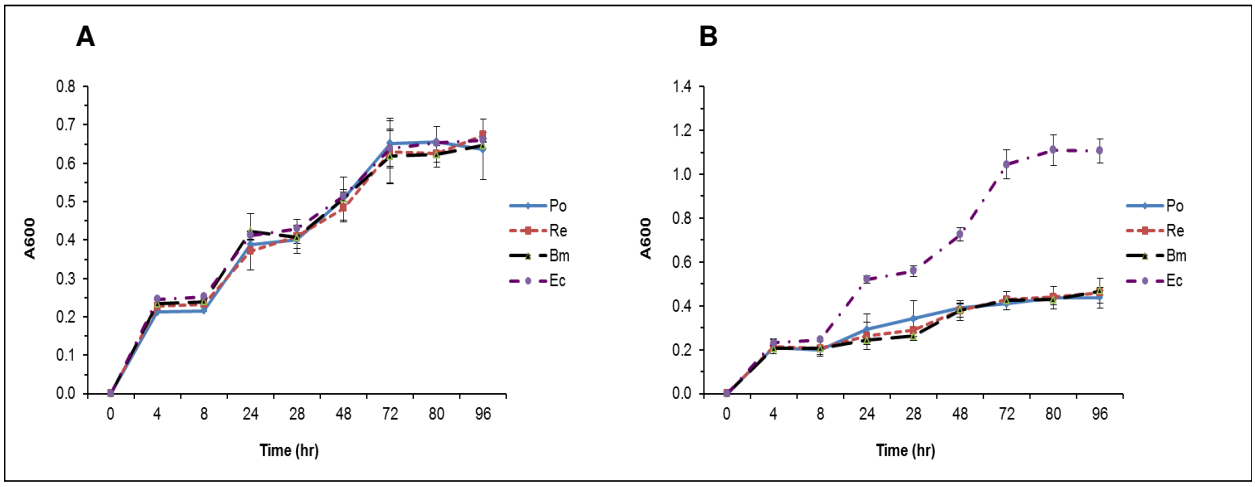

Figure 4. Effect of heptanoate on the growth profile of known PHB producing bacteria. Cultures were grown in minimal media containing 0.2% heptanoate at 30°C (panel A) and 37°C (panel B). Growth was monitored by measuring the absorbance at 600 nm as a function of time. Seeding for all cultures at time 0 h was done at an A600 of 0.02. Cultures were grown in triplicates. Po, *P. oleovorans;* Re, *R. eutropha;* Bm, *B. megaterium;* Ec, *E. coli*.

extraction of PHB granules and quantification of poly(3-hydroxybutyrate). On the basis of this assay, it is being assumed that these microbes are synthesizing PHB.

The unknown cultures were grown in minimal media containing glucose as the carbon source in the presence of 0.2% heptanoate or 0.2% malate as the substrate for five days at 30°C following which PHB was quantified. Heptanoate proved to be a better substrate than malate for PHB synthesis (Figure 6). While cultures number 3, 6, 8, 13 and 16 showed a significant increase in the PHB content, culture number 4 showed a drastic decrease in PHB levels suggesting that these particular compounds may be growth inhibitory; cultures number 10 and 16 preferred malate, while cultures number 1 and 2 did not show any substrate specific increase in PHB production.

Identification of the unknown species

Identification of the unknown species was performed by 16S rRNA sequencing using a commercial custom service (Laragen, Los Angeles). The 16S rRNA gene

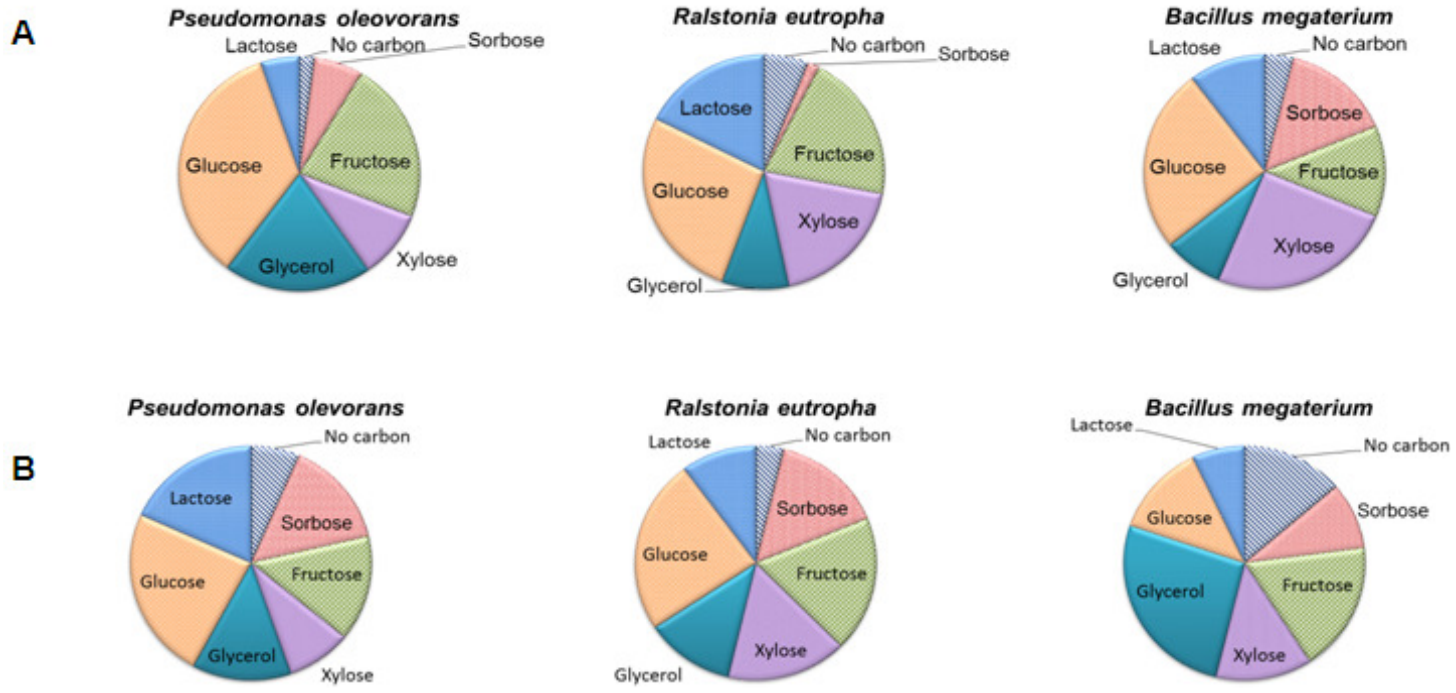

Figure 5. Effect of carbon source on PHB production in known PHB producing bacteria. Cultures were grown at 30°C for five days in 3 ml of minimal media lacking or containing different carbon sources at a concentration of 0.4% (w/v) in the absence (panel A) or presence (panel B) of heptanoate (0.2%, v/v). PHB extraction was performed as explained under materials and methods. Assays were done in triplicates.

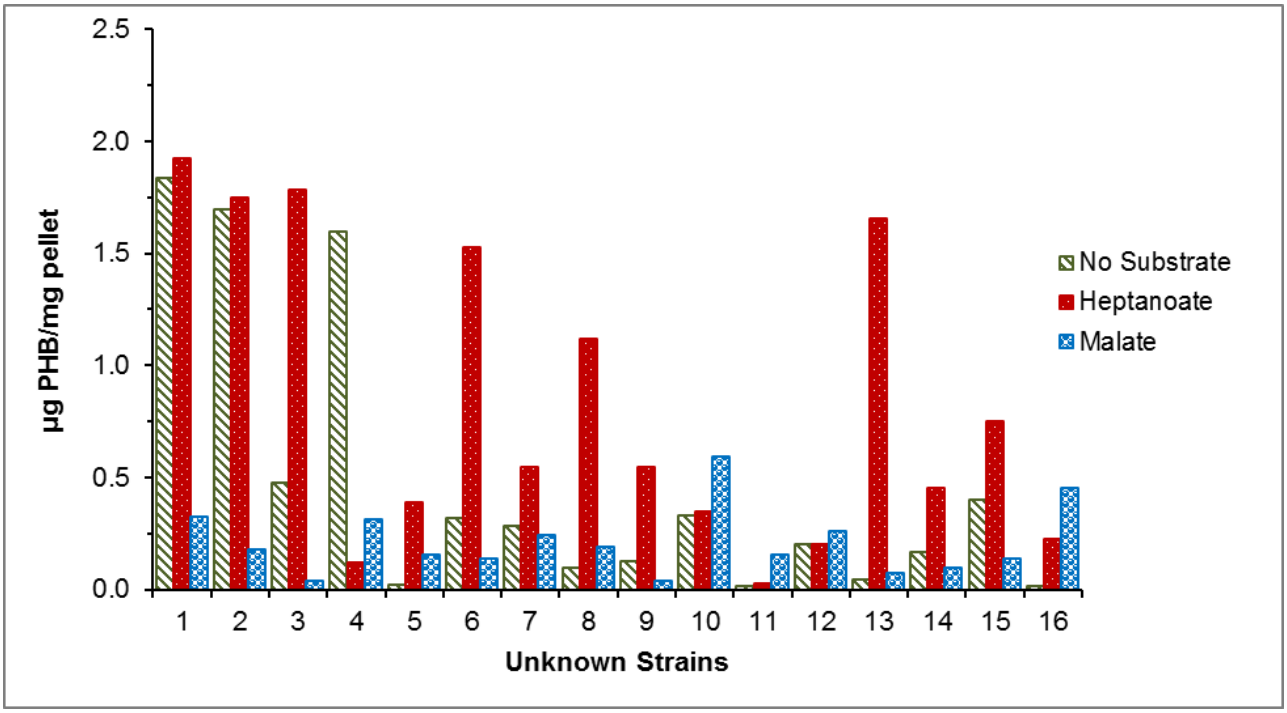

Figure 6. Production of PHB by unknown bacterial species collected from diverse environments. Microbial samples were collected from several different sites and numbered no.1 through no. 16. Cultures were grown at 30°C for five days in 3 ml of minimal media lacking or containing the substrates- 0.2% heptanoate or 0.2% malate. PHB extraction was performed as explained under materials and methods. Assays were done in triplicates.

Table 2. Identification of the microbes by 16S rRNA sequencing.

Unknown strain no.	Identification
1	Not available
2	Not available
3	Not available
4	*B. niacini PM1*
5	*Micrococcus* sp. *C-09*
6	*R. aquatilis*
7	*Arthrobacter* sp. *C-101*
8	Not available
9	*Xanthomonas* sp. *P5*
10	*Stenotrophomonas G-44-1*
11	Not available
12	*S. maltophilia AK21*
13	*S. maltophilia 13635L*
14	*Xanthomonas* sp. *P5*
15	*Staphylococcus* sp. *II B11*
16	*P. fluorescens DSS73*

from these strains was amplified by polymerase chain reaction (PCR) and 500 bp of the product was sequenced. Results obtained by sequence comparisons using the Basic Local Alignment Search Tool (BLAST) program are shown in Table 2. Several different bacteria were identified such as *Rahnella, Stenotrophomonas, Staphylococcus* and *Pseudomonas*. Three cultures, *Rahnella aquatilis* (No.6), *Stenotrophomonas maltophilia* 13635L (No. 13) and *Pseudomonas fluorescens* DSS73 (No. 16) were chosen for further studies. *R. aquatilis* is a rare gram-negative bacterium usually found in fresh water and belonging to the Enterobacteriaceae family (Harrell et al., 1989). The present work is the first report indicating that, this microbe is capable of producing PHB. *S. maltophilia* is a gram-negative bacterium commonly found in aquatic environments (Ryan et al., 2009) and whose genome was sequenced recently (Crossman et al., 2008). *S. maltophilia* has been previously shown to be capable of degrading polyhydroxybutyrate (Mergaert and Swings, 1996) although, there has been no report showing actual synthesis of polyhydroxybutyrates. Its genome sequence however, has revealed the presence of all the genes encoding enzymes involved in the PHB synthetic pathway, 3-ketoacyl-CoA thiolase (PhaA, Accession no. CAQ43773.1), acetoacetyl-CoA reductase (PhaB, Accession no. YP_001973050.1), putative polyhydroxyalkanoate synthase (PhaC, Accession no. YP_001972712.1) and PHB depolymerase (PhaZ, Accession no. YP_001971328.1). The present work is the first report to experimentally show the synthesis of PHB in *S. maltophilia* and determine its metabolic profile using Biolog panels. *P. fluorescens* is a gram-negative bacterium found in soil, water and plant surfaces and has been utilized for several bioremediation applications to degrade pollutants (Baggi et al., 1983). It has previously been experimentally shown to produce PHB (Jiang et al., 2008) and its genomic sequence indicates the presence of the PhaA/B/C/Z genes involved in PHB metabolism (Paulsen et al., 2005).

Effect of nitrogen concentration on PHB synthesis

It has been reported that, PHB production is enhanced under limiting conditions of nitrogen and phosphorus/ sulfur (Verlinden et al., 2007). In order to investigate the effect of nitrogen on the PHB synthetic capacity of these strains, cultures were grown in minimal media containing different concentrations of NH_4Cl ranging from 2 to 90 mM, in the presence of heptanoate. Both *P. oleovorans* (Figure 7A) and *R. eutropha* (Figure 7B) showed a decrease in PHB synthesis with increasing concentrations of NH_4Cl. Highest levels of PHB were observed at 2 mM NH_4Cl which is approximately $1/10^{th}$ of the concentration of NH_4Cl routinely used in minimal media (~18.5 mM). Interestingly, *R. aquatilis* and *S. maltophilia* exhibited opposite profiles. While *Stenotrophomonas* (Figure 8b) produced more PHB under limiting nitrogen conditions (highest at 2 mM), *Rahnella,* (Figure 8a) on the other hand, produced more PHB with increasing nitrogen concentrations (highest at 90 mM). In the complete absence of NH_4Cl (0 mM), no growth was observed. Thus, although PHB synthesis is enhanced under nutrient limiting conditions, certain strains may not be able to grow well at low nitrogen concentrations. Under nitrogen limited conditions (2 mM NH_4Cl) in the absence of heptanoate, PHB production was highly compromised for most of the cultures (Figure 9b). Interestingly, *P. oleovorans* produced equivalent quantities of PHB irrespective of the presence or absence of heptanoate (Figure 9a).

Detection of PHB

The newly identified PHB producing bacteria were shown to accumulate PHB via microscopic staining techniques using Sudan black B stain (Schlegel et al., 1970). The three bacteria, *R. aquatilis*, *S. maltophilia* as well as *P. fluorescens* (Figure 10 panels B, C and D, respectively) displayed reactivity in the form of intense purple granules/ cells against a safranin counter-stained background. Maximum accumulation was observed in *R. aquatilis*; no positive reaction was detected in *E. coli* (Figure 10A).

Phenotype microarray analysis

The Biolog phenotype microarray (PM) approach (Biolog Inc., Hayward, CA) is a high throughput system for the identification of carbon sources and other nutrients used for the growth of various microorganisms (Bochner et al., 2001; Bochner, 2009). Phenotype microarrays were used

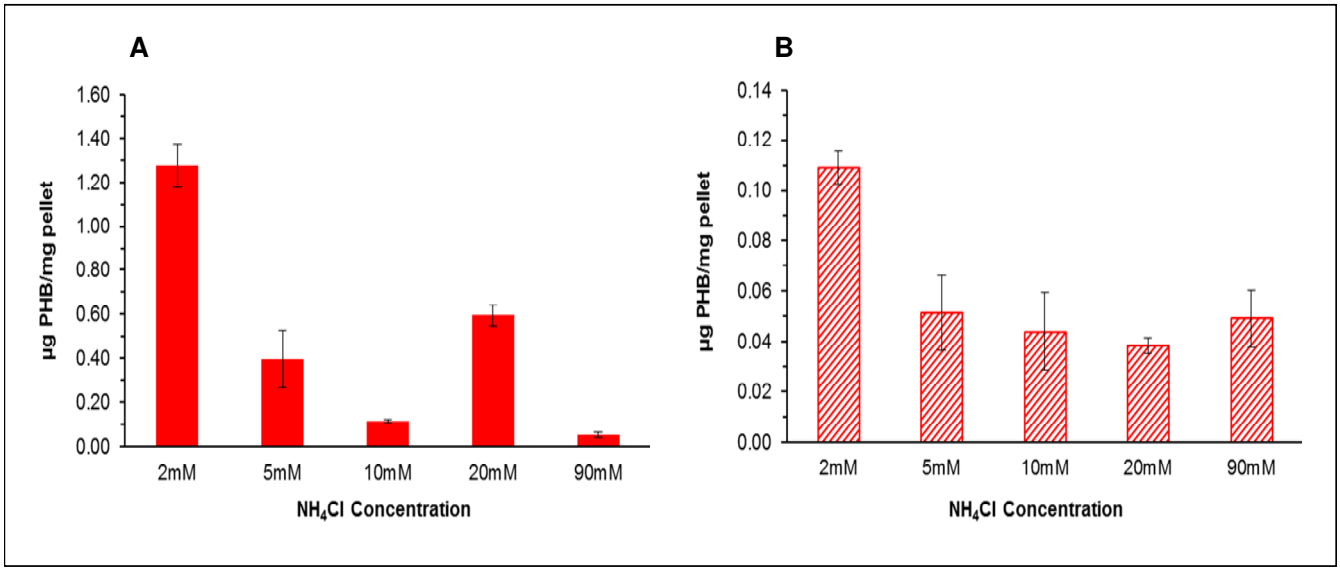

Figure 7. Effect of nitrogen limitation on the production of PHB in *P. oleovorans* and *R. eutropha.* Cultures of *P. oleovorans* (panel A) and *R. eutropha* (panel B) were grown at 30°C for five days in 3 ml of minimal media containing heptanoate (0.2%). The nitrogen source-NH_4Cl was used at different concentrations in the minimal media. PHB extraction was performed as explained under materials and methods. Assays were done in triplicates.

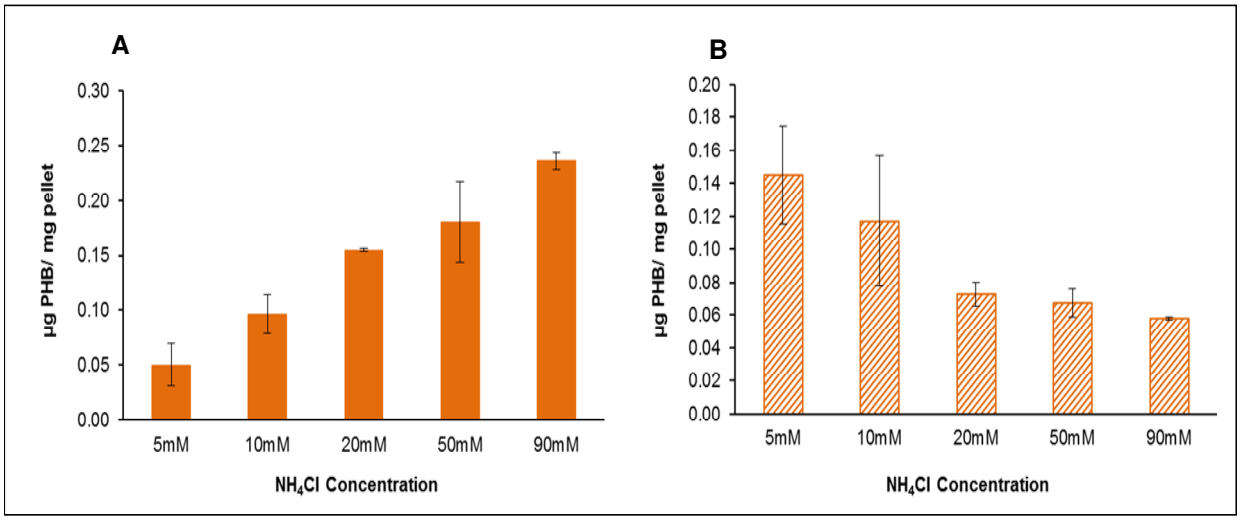

Figure 8. Effect of nitrogen limitation on the production of PHB in *R. aquatilis and S. maltophilia.* Cultures of *R. aquatilis* (panel A) and *S. maltophilia* (panel B) were grown at 30°C for five days in 3 ml of minimal media containing heptanoate (0.2%). The nitrogen source-NH_4Cl was used at different concentrations in the minimal media. Assays were done in triplicates.

to assess carbon, nitrogen, phosphorus and sulfur source utilization as well as pH profiles of the three newly identified strains, *R. aquatilis, S. maltophilia* and *P. fluorescens.* 96-well PM Biolog panels were utilized- PM1 (Carbon and energy sources), PM3B (Nitrogen sources), PM4A (Phosphorus and sulfur sources) and PM10 (pH) according to the manufacturers' guidelines. Cultures were grown for 16 h in liquid LB media and seeded onto BIOLOG plates at identical cell densities. Plates were incubated at 37°C for 24 h, following which cell viability assay was conducted and growth was monitored spectrophotometrically at A570. Color formation was recorded using a microplate reader (Biotek). This analysis was primarily performed to determine whether these strains utilize certain nutrients better than others and respond by showing an increased growth rate. The condensed version of the overall results of this analysis is shown in Table 3. Complete Biolog panel results obtained in this

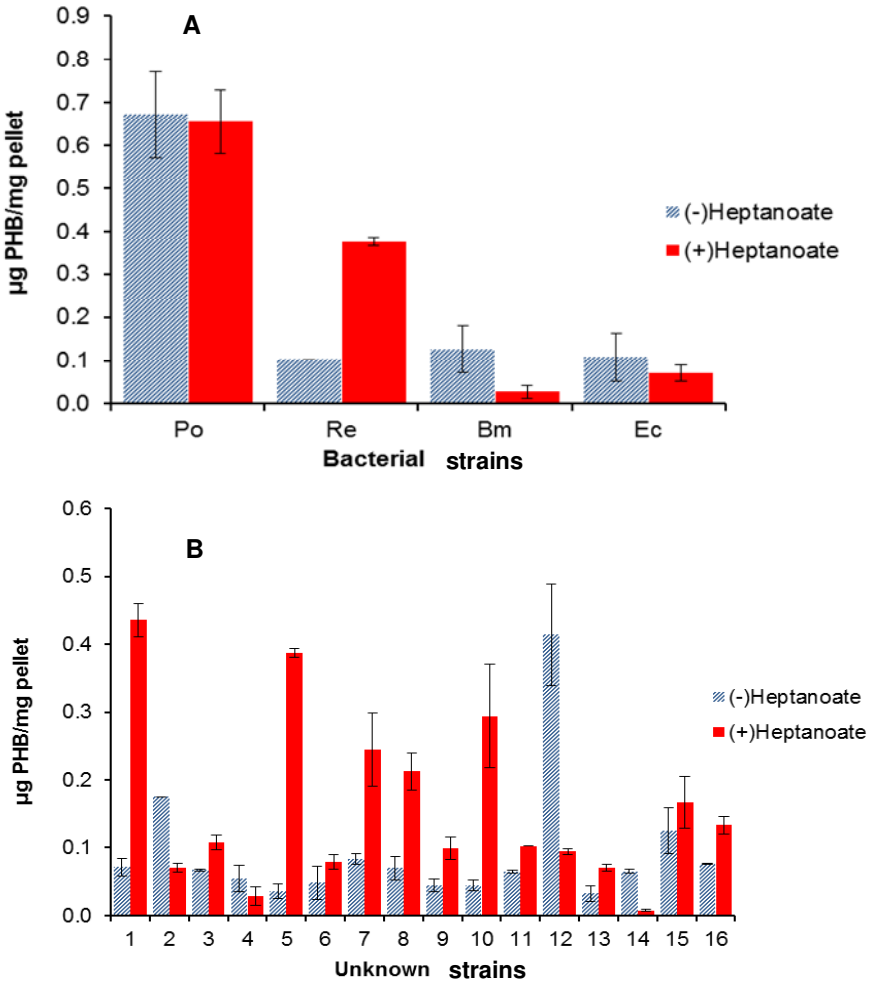

Figure 9. Combined effect of nitrogen and substrate limitation on the production of PHB in known and unknown cultures. Cultures of known (panel A) and unknown (panel B) strains were grown at 30°C for five days in 3 ml of minimal media containing 2 mM NH_4Cl in the presence or absence of heptanoate (0.2%). Assays were done in triplicates.

study are shown in online resource (Supplementary Figures 1 through 12). As an example, one of the well utilized nitrogen sources obtained from the Biolog profile-cytidine, a nucleoside consisting of cytosine and ribose sugar- was analyzed to determine its effect on PHB production on all strains used in this study (Figure 11). Cytidine has previously been shown to function as a nitrogen source in several *Pseudomonas* species (Chu and West, 1990; West, 1994). It is our goal to furtherutilize the phenotype microarray panels in the future and comprehensively study these strains for finer optimization of conditions under which higher quantities of PHB can be obtained.

DISCUSSION

Although, previous research has shown that a large number of bacterial species, both gram positive and gram negative, produce PHBs (Verlinden et al., 2007) as well as degrade them (Mergaert and Swings, 1996), the sheerdiversity of the microbial world calls for the identification of bacteria capable of producing large amounts of PHB utilizing cheap nutrient sources. This requires careful optimization and analyses of conditions under which PHB synthesis is maximized. High production costs have not enabled the full commercialization of bioplastics although, a number of strategies such as the use of recombinant microbialstrains, more efficient fermentation processes and recovery/purification protocols and the use of inexpensive substrates have been adopted to bring down the production costs (Verlinden et al., 2007). At the same time, the vast potential to screen for novel bacterial species capable of producing high levels of PHB remains untapped, although, some reports have examined the

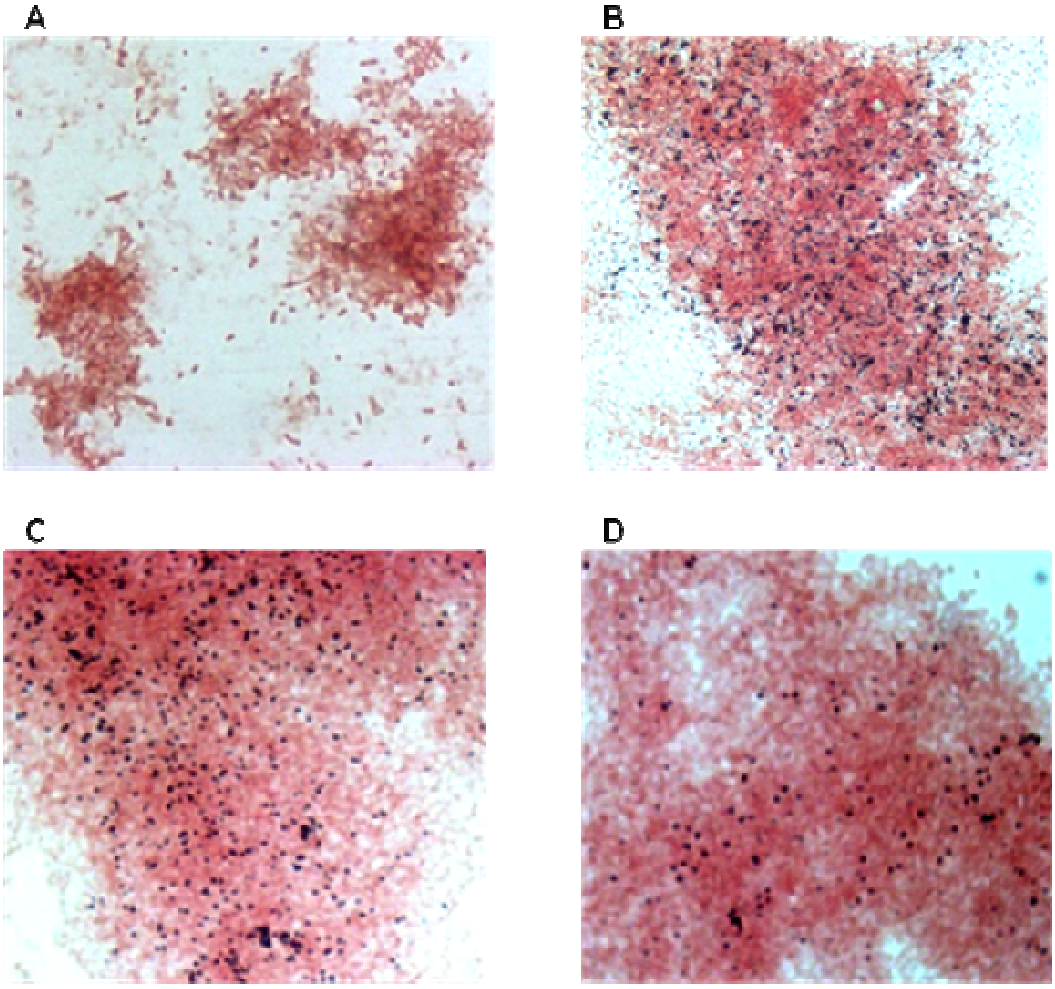

Figure 10. Micrographs of bacteria showing PHB accumulation. Cultures of *E. coli* (panel A), *R. aquatilis* (panel B), *S. maltophilia* (panel C) and *Pseudomonas* (panel D) were grown in minimal media containing heptanoate for 5 days. Smears prepared on glass slides were stained with Sudan black and counter-stained with safranin. Imaging was performed under bright-field illumination at 1000x magnification.

presence of PHB producing bacteria in diverse environments such as marine microbial mats present in cannery waste streams (Lopez-Cortes et al., 2008), oil contaminated soils in oil fields (Yao et al., 1999), activated sludges from pesticide and oil refineries effluent treat-ment plants (Porwal et al., 2008) and salty waters (Rehm, 2003). In the present paper, we have successfully isolated and characterized novel species of PHB producing bacteria present in routinely used public areas (such as beaches) and physical objects (such as children's' plastic toys). The PHB synthesizing capacity of these bacteria was compared with that of the mostly widely studied PHB producing strains such as *R. eutropha* (Khanna and Srivastava, 2005), *P. oleovorans* (Yao et al., 2009) and *B. megaterium* (Kulpreecha et al., 2009). A number of researchers have explored the nutrient requirements of some of these strains for optimizing PHB synthesis.

Our study is the first to report that, the carbon source preference of these strains changes depending on the presence or absence of the PHB substrate. Our finding of heptanoate being the most preferred substrate corroborates the findings of Yao et al. (1999) who showed that, a variety of *Pseudomonads* produce PHB by utilizing hexanoate and octanoate as substrates. In addition, we were also able to show that the presence of a substrate does not impact the growth rate of the PHB producing bacteria and hence, biomass is not likely to be compromised. Based on the PHB assays, the unknown bacteria were identified by partial 16S rRNA sequencing which indicated the predominant presence of gram-negative species, specifically *S. maltophilia.*

Table 3. Phenotype microarray analyses of *R. aquatilis*, *S. maltophilia* and *P. fluorescens.*

Biolog PM panels	*R. aquatilis*		*S. maltophilia* 13635L		*P. fluorescens* DSS73	
	Growth enhancers	Growth inhibitors	Growth enhancers	Growth inhibitors	Growth enhancers	Growth inhibitors
Carbon sources	D-Trehalose 2-Deoxy Adenosine Inosine	Tween-20 D-Aspartic acid 1,2 -Propanediol M-Inositol Glyoxylic acid L-Lyxose	D-Trehalose 2-Deoxy Adenosine Inosine D- fructose Glucuronic acid Galacuronic acid	Tween 20 D-Aspartic acid 1,2 -Propanediol M-Inositol Tyramine D-Threonine	D-Trehalose 2-Deoxy Adenosine Inosine D-Melibiose D-Cellobiose Fructose-6-Phosphate	Tween 20 D-Aspartic acid 1,2 -Propanediol M-Inositol Glyoxylic acid L-Lyxose
Nitrogen sources	Cytidine Ala-Asp Gly-Glu Adenosine Gly-Asn	Nitrate D-Glutamic acid Histamine Guanine Inosine Urea Ethylenediamine	Cytidine Adenosine Gly-Glu L-Glutamic acid D-Glucosamine	Nitrate Histamine Inosine	Gly-Glu D- Glucosamine D-Aspartic acid Ala-Asp Cytosine N-Acetyl-D-Glucosamine	Nitrate D-Glutamic acid Guanine Inosine Uric acid Nitrite D-Lysine
Phosphorus and sulfur sources	Glycyl -L - Methionine O-Phospho -D-Serine Phosphoenol Pyruvate Guanosine - 3’ - monophosphate phosphate	D-Glucose-6-Phosphate Tripolyphosphate Uridine - 2’ - monophosphate Uridine - 3’ - monophosphate	Trimetaphosphate D-Glucose-1-phosphate D-Glucosamine-6-Phosphate Guanosine - 3'-monophosphate	Tripolyphosphate D, L-Ethionine Adenosine - 5’ - monophosphate	Cysteamine O-Phospho -L-Threonine Adenosine-3'-monophosphate Guanosine-3'-monophosphate	Tetramethylene sulfone L-Djenkolic acid 2-Hydroxyethane Sulfonic acid
pH	pH 6 pH 4.5 + Methionine pH 9.5 + Alanine X-Caprylate X-α-D Galactoside		pH 6 pH 9.5+ Tyrosine pH 9.5 + Urea pH 4.5+ Methionine		pH 6, 7 pH 9.5 + Tyrosine	pH 4.5 + Methionine

Interestingly, amongst the PHB producers detected, our study is the first to demonstrate PHB synthesis by a novel species, *R. aquatilis*, which previously not been studied. In addition, although, the genome of *S. maltophilia* doescontain the genes involved in PHB metabolism, this is the first report to experimentally show the synthesis of PHB by this microbe. It will be worthwhile to exploit this species further and optimize its PHB production capacity via different fermentation strategies. Although, a large amount of experimental work

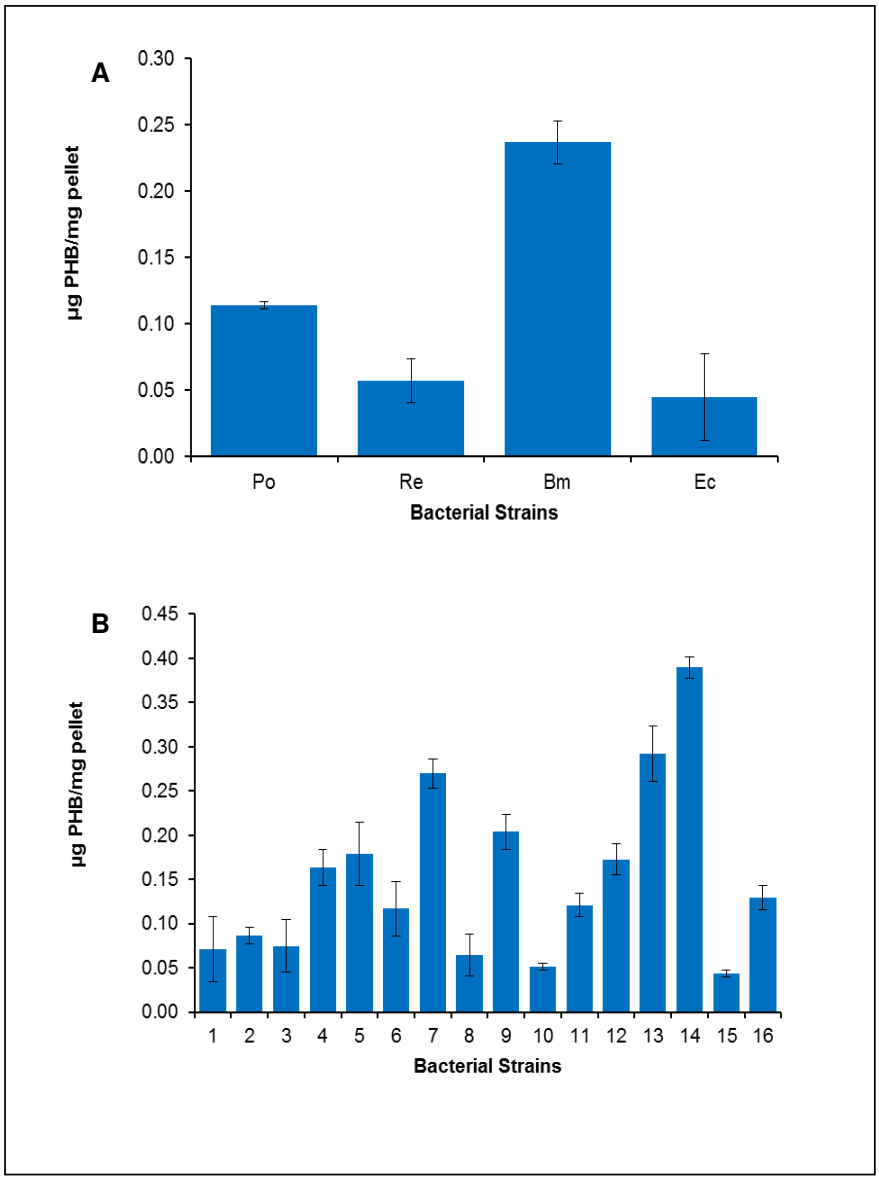

Figure 11. Effect of cytidine on the production of PHB in known and unknown cultures. Cultures of known (panel A) and unknown (panel B) strains were grown at 30°C for five days in 3 ml of minimal media.

needs to be done before these two novel organisms can be utilized commercially, it is nevertheless an exciting finding and demonstrates the need to study bioplastic producing bacteria further.

Bacteria have been found to accumulate PHBs when there is a limitation of nutrients, specifically nitrogen sources (Steinbuchel and Fuchtenbusch, 1998). In this study, we were able to show that this finding cannot be generalized for every PHB producing bacteria. While *S. maltophilia* did follow the expected trend, *R. aquatilis*, on the other hand, showed a decrease in PHB synthesis with decreasing concentrations of ammonium chloride in the media. This leads us to hypothesize that PHB may be synthesized by different microbes depending on their individual physiological requirements and that in certain species, PHB accumulation may not truly occur in response to nutrient stress as has been generally accepted. More detailed studies need to be performed before this statement can be validated. Our study also shows that, decrease in nitrogen levels alone are not enough to produce significant levels of PHB and that a substrate is required nevertheless.

To further characterize the two novel PHB producing species that have been described in this work, phenotype microarray (PM) analysis was performed. These analyses were primarily done to determine conditions under which

these microbes would display maximum growth and then, utilize those conditions to test for PHB production. These panels are very useful as they allow the analysis of a large number of cellular properties simultaneously. The panels used in this study, carbon, nitrogen, phosphorus/ sulfur sources as well as pH, were instrumental in providing preliminary information about nutrients which enhance or impede growth of these strains. A follow-up study is being designed to pursue optimization of PHB synthesis using the extensive data that has emerged from this study, which may provide valuable insights into the production of PHB by novel bacteria using cheap nutrient sources.

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