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Full Length Research Paper

Influence of substrate concentration in accumulation pattern of poly(R) hydroxyalkonoate in *Pseudomonas putida* SU-8

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Polyhydroxyalkanoates (PHAs) are accumulated by many bacteria intracellularly as carbon and energy reserves in presence of excess carbon source and limited nitrogen or other nutrient sources. The accumulation pattern of these PHA may vary as the supplements are varied. In this study, *Pseudomonas putida* SU-8 was allowed to grow in glucose and lactose containing medium, the organism was found to accumulate more PHA when the nitrogen source was limited. The organism was found to accumulate PHA at the early log phase when it was allowed to grow in nitrogen limited medium. The organism did not accumulate PHA when it was limited with phosphate source. It was also found to accumulate homopolymer PHB while growing in either glucose or lactose containing medium.

Key words: *Pseudomonas putida* SU-8, polyhydroxyalkonoate, polyhydroxybutyrate, fourier transform infrared spectroscopy.

INTRODUCTION

Poly (3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules accumulated as insoluble granules in the cytosol of Gram negative and Gram positive bacterial cell (Anderson and Dawes, 1990; Doi, 1995; Lemoigne, 1925). Sometimes, these granules may be accounted for more than 80% of the cellular dry weight. The microorganisms have developed various strategies to accumulate PHAs, as the accumulation is found higher when the organisms are grown in excessive carbon source and limited other nutrients sources such as nitrogen, phosphorus and sulphur (Kessler and Witholt, 1999). PHAs are natural polyesters which are recyclable not like the petrochemical thermoplastics and finds application in many fields.

PHAs are subdivided into three broad classes according to the size of comprising monomers. PHAs containing up to C5 monomers are classified as short chain length PHAs (scl-PHA), C6–C14 as medium chain length (mcl-PHA) and C14 monomers as long chain length (lcl-PHA) PHAs (Madison and Huisman, 1999). Among these PHAs, scl-PHAs have properties close to conventional plastics while the mcl-PHAs are regarded as elastomers and rubbers. PHB is the most common type of scl-PHA and this homopolymer of 3-hydroxybutyric acid has been studied most extensively. It was found that alteration in substrate influences change in homopolymer or copolymer of PHA accumulation by the organism (Samrot et al., 2011). Copolymers of PHA can be formed containing either two or more of the monomers of 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV), 3-hydroxyhexanoate (3-HH) or 3-hydroxyoctanoate (3-HH) or 4-hydroxybutyrate (4HB) (Samrot et al., 2011; Otari and Ghosh, 2009). *Pseudomonas oleovarans* was found to accumulate PHA consisting of medium chain

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Abbreviations: **PHA**, Polyhydroxyalkonoate; **PHB**, polyhydroxybutyrate; **FTIR**, fourier transform infrared spectroscopy.

3-hydroxy alkonic acid in alkanes, alkanols and alkonic acids (Brandl et al., 1988; Lageveen et al., 1988; de Smet et al., 1983). Some *Pseudomonas* sp involves in the synthesis of co-polyesters consisting of medium chain 3-hydroxy alkonic acids from acetyl coA (Brandl et al., 1988; Lageveen et al., 1988). This pathway has not been studied in detail. *Pseudomonas stutzeri* 1317 was found to accumulate mcl-PHA from unrelated carbon sources and glucose (He et al., 1998). Most of the microbes synthesize either scl-PHAs containing primarily 3HB units or mcl-PHAs containing 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD) as the major monomers (Brandl et al., 1988; Anderson and Dawes, 1990; Steinbüchel and Valentin, 1995; Steinbuchel, 1995) PHA accumulation in *P. putida* CA-3 was found to be increased as nitrogen was depleted in the medium (Ward et al., 2005).

This study was done to find out the effect of carbon, nitrogen, phosphate sources and their concentrations on PHA accumulation pattern in *P. putida* SU-8.

MATERIALS AND METHODS

Isolation of the bacterial strain

The bacterial strain was isolated from the garden soil of Sathyabama University, Chennai, Tamil Nadu - 600119, India. Soil sample collected was incubated in 50 ml of Luria Bertani (LB) medium for several hours. After incubation, 0.5 ml of the supernatant was inoculated into minimal media containing KH_2PO_4 (3 g/L), Na_2HPO_4 (6 g/L), NH_4Cl (2 g/L), NaCl (5 g/L) and Mg SO_4 (1g/L). After 2 days of cultivation at 37°C, 0.5 ml of the culture broth was diluted with 50 ml of minimal media (100- fold dilution). These procedures were repeated for three times in order to stimulate an enrichment culture. After the enrichment steps, the culture broth was spread out on minimal media solidified with 2% agar. This strain was stored in minimal media with 25% glycerol at -70°C. The isolated culture was subjected for further study.

Identification of microorganism

Identification of microorganism was done by performing routine biochemical tests and 16S rRNA sequencing (Pitcher et al., 1989). 16S rRNA sequencing was done by isolation of DNA from the organism and the large fragment of the 16S rRNA gene was amplified by PCR using the universal primers BAC-F-(5'-AGA GTT TGA TC(AC) TGG CTC AG-3') BAC-R (5'AAG GAG GTG (AT)TC CA(AG) CC-3'). The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, USA) according to the manufacturer's instructions. The PCR product after purification is sequenced using a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and a model 3100 automatic sequencer (Applied Biosystems, USA). The closest known relatives of the new isolates were determined by performing a sequence database search. The sequences of closely related strains were retrieved from GENBANK and the Ribosomal Database Project (RDP) libraries.

Production and optimization of PHA

The bacterial isolate was inoculated into minimal media containing Potassium dihydrogen phosphate (3 g/L), disodium hydrogen phosphate (6 g/L), ammonium chloride (2 g/L), sodium chloride (5 g/L) magnesium sulphate (1 g/L) and a carbohydrate source (either lactose or glucose). The production of PHA was optimized with respect to different carbon sources such as glucose and lactose at various concentrations. The optimum conditions like incubation time, pH and temperature for PHA accumulation were determined. Other optimization parameters such as limitation of nitrogen source and other factors were also done.

Isolation of PHA

PHA was isolated following the modified method of Law et al. (2001). The fermented culture was centrifuged at 14,333 g for 25 min at 4°C and washed with distilled water and freeze dried. One gram of the freeze dried cell powder was treated with a dispersion containing 15 ml of each chloroform and 30% sodium hypochlorite solution. The mixture was incubated at 37°C with 250 rpm agitation for 1 h. It was centrifuged at 2610 g for 15 min and this resulted in three layers. Upper layer contains sodium hypochlorite solution, middle layer contains non-PHA and undisrupted cells and bottom layer contains chloroform- containing PHA. The bottom layer was filtered and allowed to concentrate by evaporation to a final volume of 5 ml. Pure PHA was obtained by non solvent precipitation (Chloroform: methanol at a ratio of 1:9). Finally the white precipitate was dried and weighed.

Determination of percentage PHA accumulation

The percentage of PHA accumulation in cells was calculated by the ratio between the weight of extracted PHA to the cell dry weight.

Analytical methods to determine cell growth

Cell growth was monitored by measuring the optical density of the culture broth at regular time interval at 660 nm.

FTIR analysis

The extracted PHA was dissolved in chloroform and Infrared spectra (IR) (4000 to 400 cm^{-1}) were recorded on polymer films cast from chloroform solution onto KBr plates by using FTIR (Shimadzu, DR-800) at 27°C.

RESULTS

The bacterium was isolated and identified as *Pseudomonas putida* SU-8. The organism was found to possess novel 16S rRNA sequence and it was deposited in GENBANK (accession number: HQ640945). *Pseudomonas putida* SU-8 was grown for 48 h in minimal media containing different concentration of either glucose or lactose (1 to 6%). The organism was found to accumulate higher concentration of PHA at pH 7, 24 h and 30°C that is, 36% in glucose containing media and 35% in lactose containing media (Figures 1 to 4).

The bacterium was found to accumulate higher concentration of PHA in, i.e. 85.6% in 5% glucose containing medium with limited nitrogen source (Figure 5). PHA accumulation was found to be reduced when the other

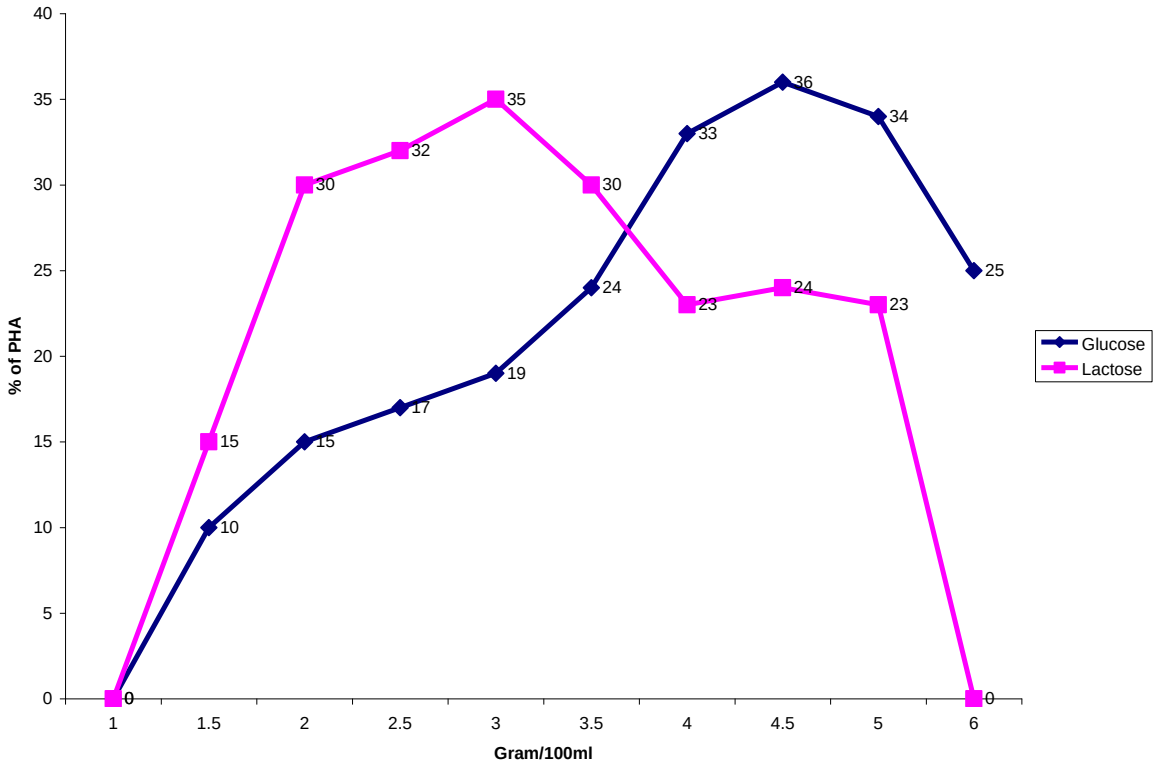

Figure 1. Accumulation of PHA by *Pseudomonas putida* SU-8 at various concentration of glucose and lactose.

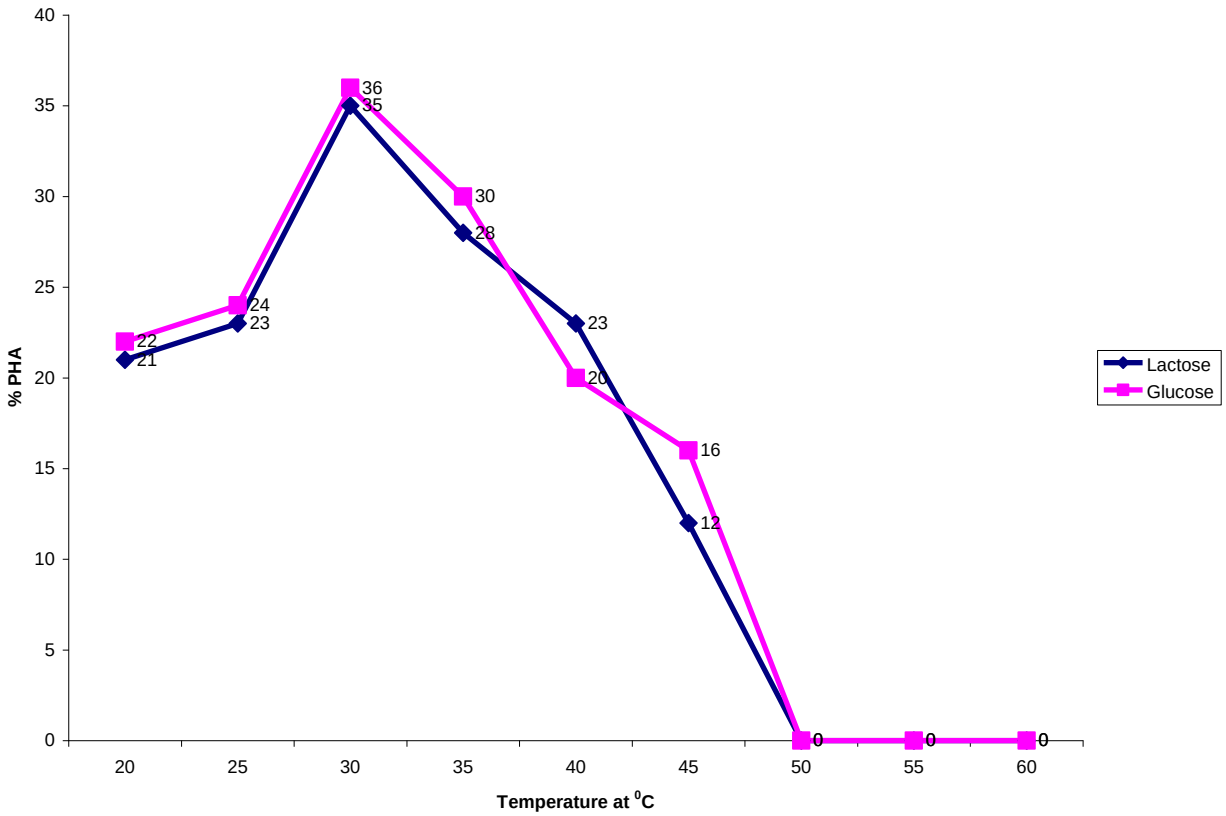

Figure 2. PHA accumulation by *Pseudomonas putida* SU-8 at various temperature.

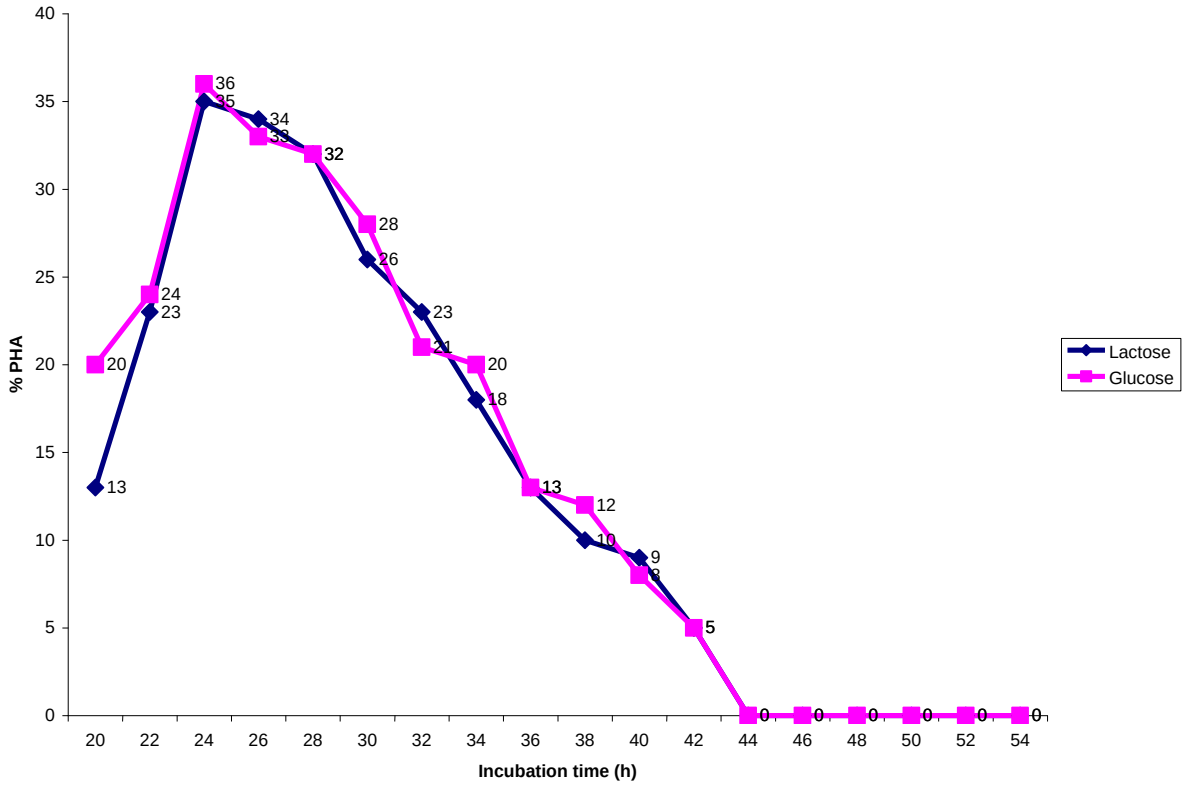

Figure 3. PHA accumulation by *Pseudomonas putida* SU-8 at various incubation time.

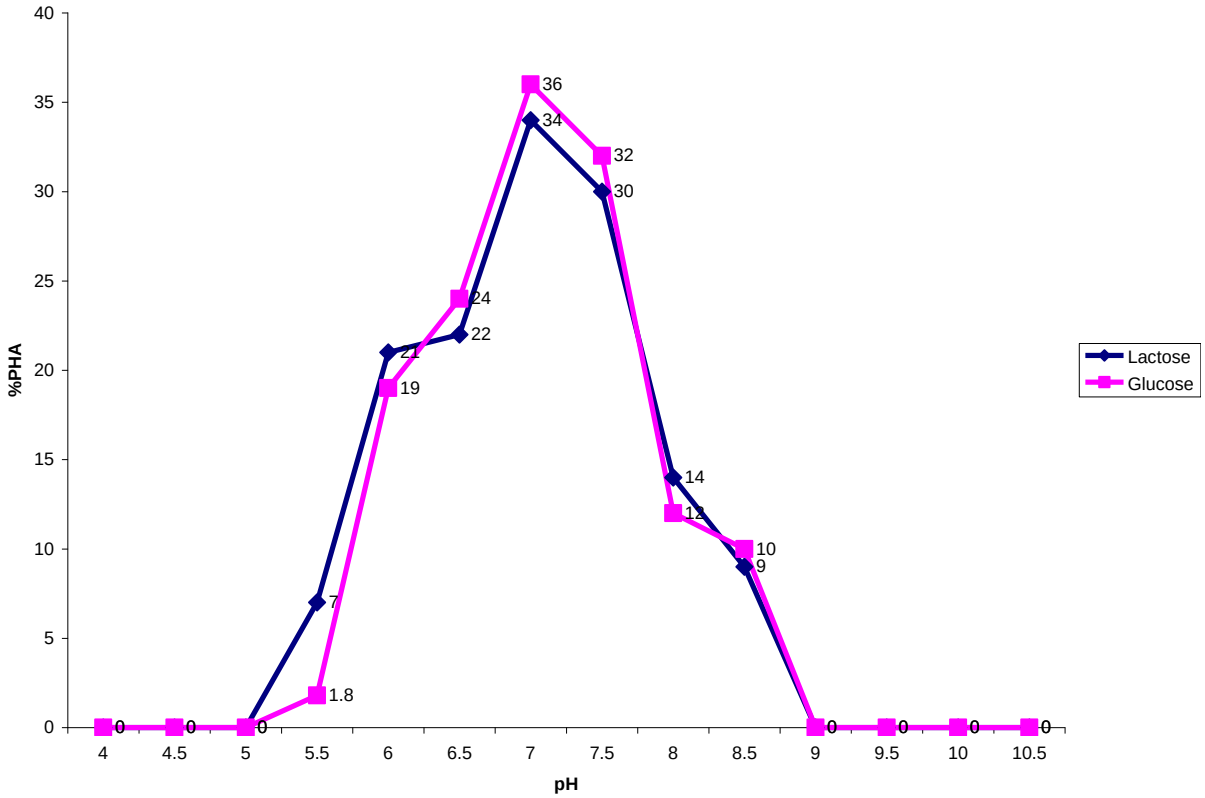

Figure 4. PHA accumulation by *Pseudomonas putida* SU-8 at various pH.

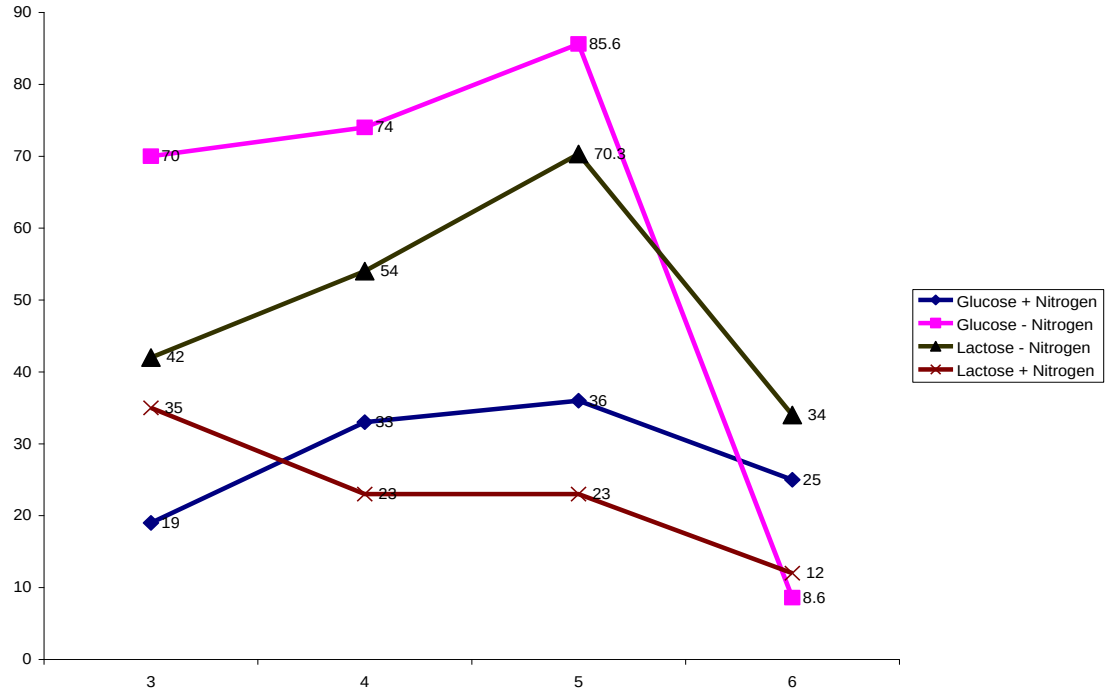

Figure 5. PHA accumulation at different concentration of carbon source and nitrogen source limited.

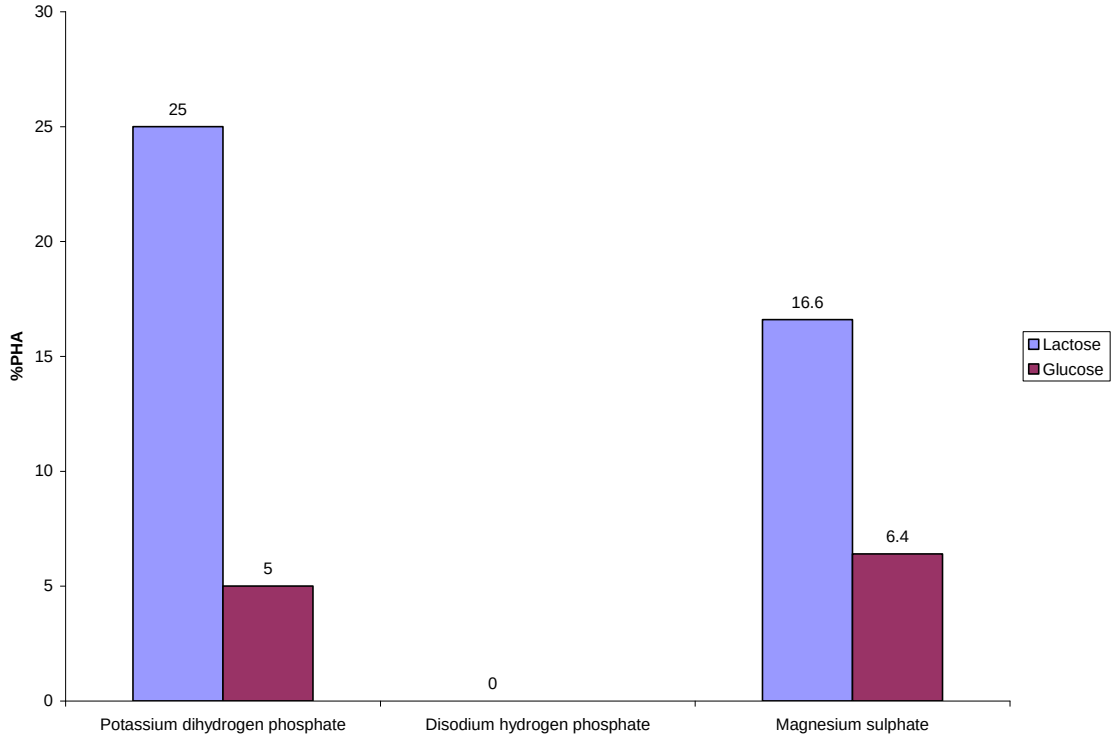

Figure 6. PHA accumulation while medium limited with any one source.

ingredients such as KH_2PO_4, Na_2HPO_4 and $MgSO_4$ (Figure 6).

PHA accumulation was found to be at early log phase of growth, when the isolate was allowed to grow in nitrogen limited medium with either lactose or glucose (Figures 7 and 8).

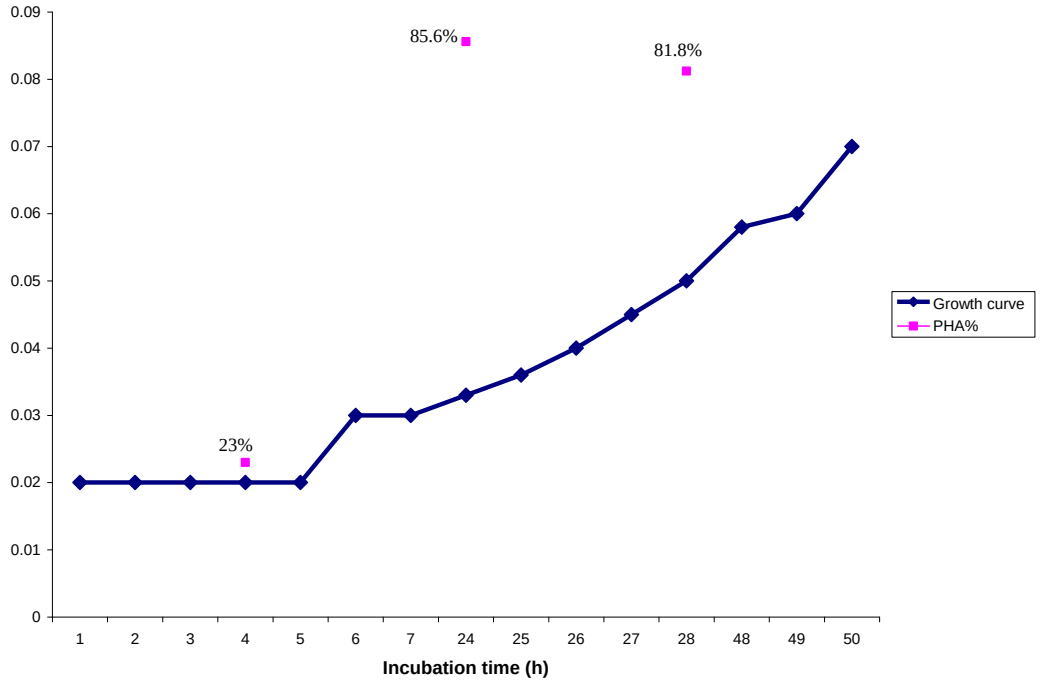

Figure 7. Accumulation of PHA vs Growth curve by *Pseudomonas putida* SU-8 in glucose containing nitrogen limited medium.

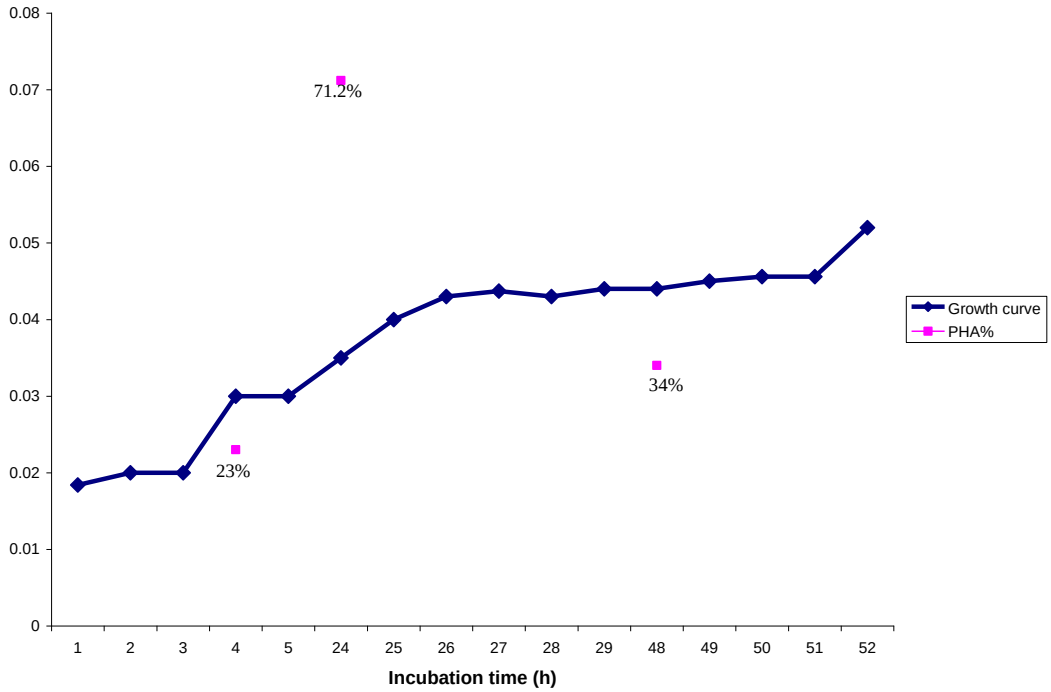

Figure 8. Accumulation of PHA vs Growth curve by *Pseudomonas putida* SU-8 in lactose containing nitrogen limited medium.

FTIR analysis confirmed the presence of PHB polymers is present in the organism grown in glucose and lactose containing medium (Figure 9). There was a strong absorption at 1293 and 1216 cm^{-1} in the PHA isolated from the organism grown in glucose which is characteristics for ester bonding. Other absorption bands

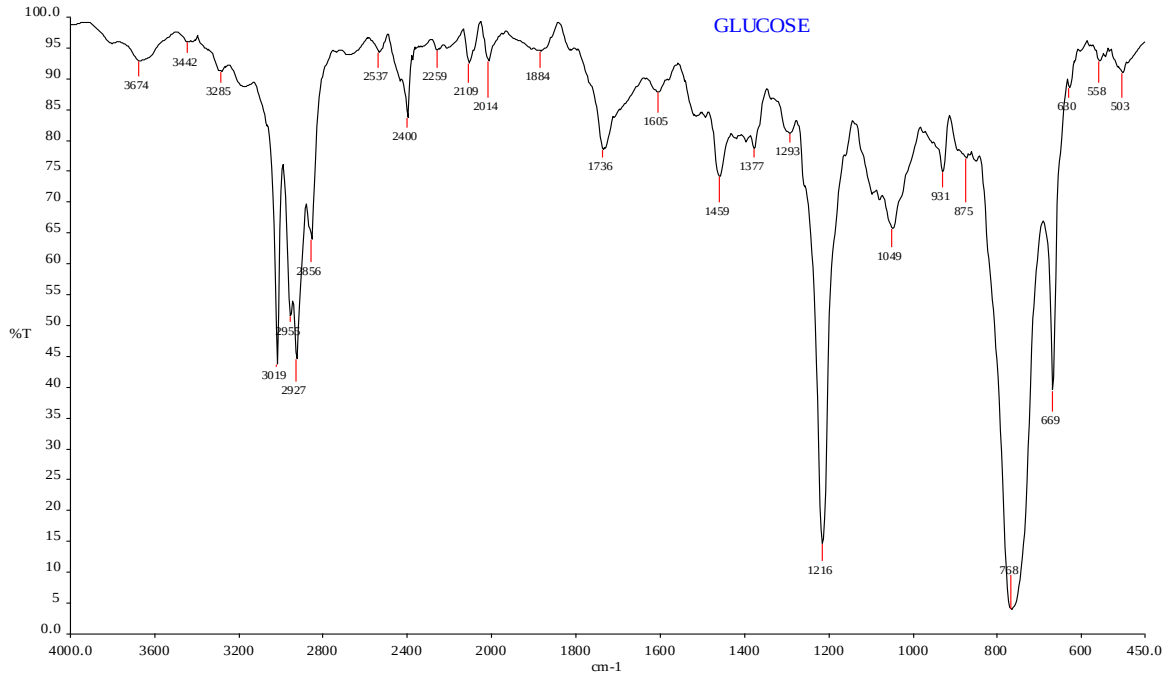

Figure 9. FTIR analysis *Pseudomonas putida* SU-8 grown in glucose containing medium.

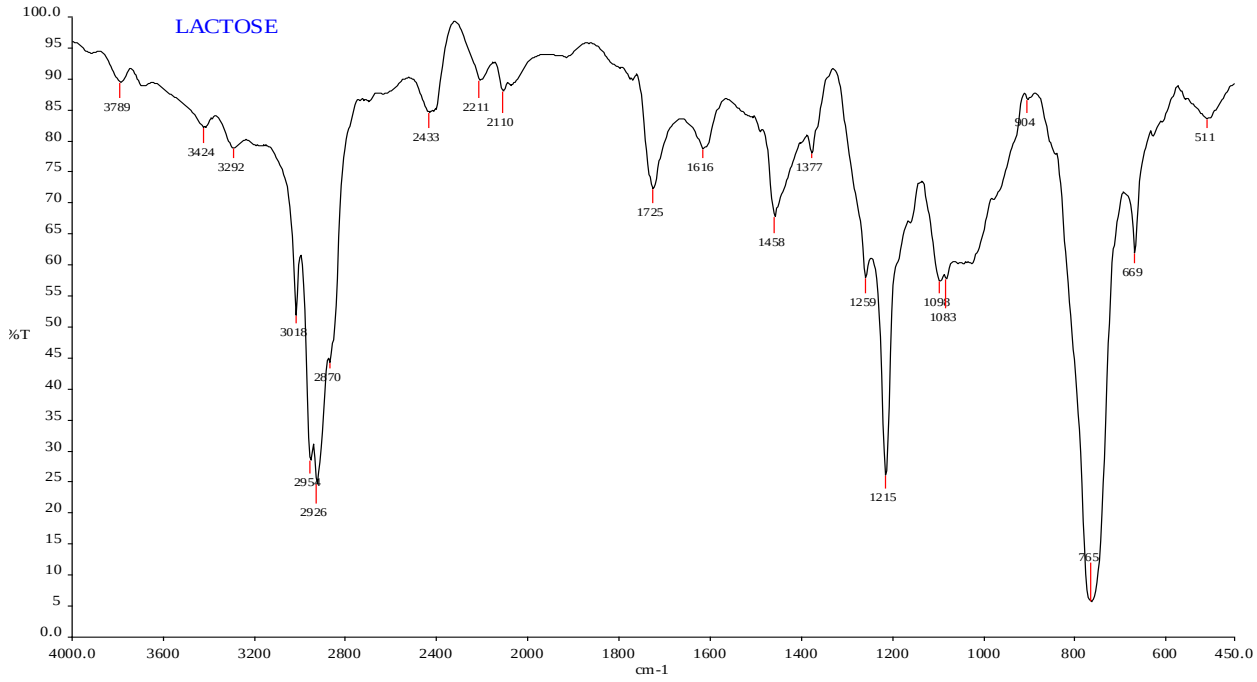

Figure 10. FTIR analysis *Pseudomonas putida* SU-8 grown in lactose containing medium.

at 1377, 1459, 1736, 2927 and 3789 cm^{-1} for CH_3, -CH_2, C=O, -CH and OH groups respectively. Even in the FTIR analysis of PHA isolated from the organism grown in lactose containing medium, there was a strong absorption in at 1279 cm^{-1} which is characteristics for ester bonding. Other absorption bands at 1377, 1458, 1724, 2926-2954 and 3789 cm^{-1} for CH_3, -CH_2, C=O, -CH and OH groups respectively. FTIR analysis of polymers isolated from lactose containing medium was also found to show polymers 3-hydroxy butyrate (PHB) (Figure 10).

DISCUSSION

Microorganisms was isolated and identified by their 16SrRNA sequence as *P. putida* SU-8. *P. putida* SU-8 was found to accumulate more PHA in glucose containing medium i.e. 36% in glucose containing media at pH 7, 24 h and 30°C and 35% PHA in lactose containing media at pH 7, 24 h and 30°C (Figures 1 to 4).

P. putida SU-8 was found to accumulate higher concentration of PHA in that is, 85.6 in 5% glucose containing medium with limited nitrogen source and 70.3 in 5% lactose containing medium with limited nitrogen source (Figure 5). Tobin et al. (2007) also found *P. putida* CA-3 to accumulate medium-chain-length polyhydroxyalkanoate (mclPHA) concurrently under nitrogen limitation. Other nutrient ingredients like potassium dihydrogen phosphate, disodium hydrogen phosphate and magnesium sulphate are also found to influence the PHA accumulation (Figure 6), which is on par with the result of Samrot et al., (2011), who found the similar result with *Enterobacter cloacae* SU-1. *P. putida* SU-8 took a longer time to grow in the nitrogen free medium, later it grew and the PHA accumulation was found to be higher when the growth phase of *P. putida* SU-8 entered early log phase (Figures 7 and 8).

FTIR analysis confirmed the presence of PHB polymers is present in the organism grown in glucose and lactose containing medium as there were strong absorption for the isolated PHA for CH_3, $-CH_2$, C=O, -CH and OH groups. FTIR analysis of polymers isolated from both glucose and lactose containing medium was found to be polymers 3-hydroxy butyrate (PHB) (Figures 9 and 10).

P. putida SU-8 was found to accumulate PHB, using the usual pathway how all the organisms stores the carbon source. The precursors for the synthesis of medium chain length (mcl) PHA are R-3-hydroxyacyl-CoA in *P. putida* (Rehm, 2006). R-3-hydroxyacyl-CoA is synthesized from the denovo biosynthesis pathway of fatty acids or β-oxidation of fatty acids and the intermediates are diverted towards PHA biosynthesis, where transacylase and enoyl CoA hydratase catalyses the process, thus, 3-hydroxyacyl-CoA act as substrate for the synthesis of medium chain length PHA (mcl-PHA) (Fukui et al*.,* 1998). The *Enterobacter cloacae* SU-1 also found to choose the same pathway like *P. putida* to produce PHO and PHH co-polymers (Samrot et al*.,* 2011).

Conclusion

P. putida SU-8 was found to accumulate higher concentration of PHA when the nitrogen source was limited. The organism was found to accumulate PHA at early log phase of the growth when it was limited with nitrogen source. The FTIR analysis of PHA of *P. putida* SU-8 grown in glucose showed that the monomers present in the polymers are PHB.

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