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Accumulation of Poly[(*R*)-3-hydroxyalkanoates] in *Enterobacter cloacae* SU-1 During Growth with Two Different Carbon Sources in Batch Culture

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Abstract Polyhydroxyalkanoates (PHAs) are polymers of hydroxyalkanoate, which are accumulated by many bacteria as food storage material under excess carbon source and limited nitrogen source. In our study, *Enterobacter cloacae* SU-1 isolated from the rhizospheric soil of *Arachis hypogea* was allowed to grow as batch culture in minimal media containing either glucose or lactose, and the pattern of PHA accumulation by *E. cloacae* SU-1 was studied. *E. cloacae* SU-1 was found to accumulate 94% of PHA/dry weight of the organism in 8 g/l lactose-containing medium. When the monomeric units of PHA of *E. cloacae* SU-1 was analyzed by gas chromatography, it was also found to accumulate medium chain length PHA 3-hydroxyoctanoate (3HO)/3-hydroxyhexanoate (3HH) in the presence of glucose and lactose, but the ratio of these monomers differed as 11:1 and 6:1, respectively.

Keywords Polyhydroxyalkanoates (PHA) · 3-hydroxyhexanoate (3HH) · 3-Hydroxyoctanoate (3HO) · *Arachis hypogea* · *Enterobacter cloacae* SU-1 · Gas chromatography (GC)

Introduction

PHA is biodegradable and has thermoplastic and elastomeric properties, which can replace the petrochemical plastics [1]. Many microorganisms are found to accumulate polyhydroxyalkanoates (PHAs), when essential nutrients become limited but have excess carbon source [2, 3]. Generally, PHA accumulation is observed during the stationary phase of the growth and be utilized in decline phase. These polyesters (PHAs) can be divided into two groups based as short chain length PHA (Scl-PHA) (three to five carbon atoms) and medium chain length PHA (Mcl-PHA) (six to 14 carbon atoms). PHA synthases are the key enzymes for the biosynthesis and are involved in the conversion of 3-hydroxyacyl-CoA substrates to PHA with the concomitant release of CoA. PHA synthases are broadly

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classified into three types based on their substrate specificity and sub-unit composition [4]. Among these, type II PHA synthases incorporates preferentially 3-hydroxy fatty acids of Mcl (C6–C14) into PHA [5]. The substrates for type II PHA synthases are derived from intermediates of fatty acid beta-oxidation [5, 6] and from fatty acid denovo biosynthesis [7]. Medium chain length PHA possesses less crystalline than short chain length PHA. Mcl-PHA also has wide medical applications than short chain length PHA [8]. A variety of copolymers of PHA have been isolated from bacteria by varying the combination of carbon substrates for growth. But, only a few reports are available regarding the production of copolymer using simple sources or a single source of carbon [9]. At present, 150 different PHA constituents are characterized [10].

In this study, PHA accumulation pattern of *Enterobacter cloacae* SU-1 in lactose- and glucose-containing medium was studied, and the monomeric units of the polymer was analyzed by gas chromatography (GC).

Materials and Methods

Isolation of the Bacterial Strain

The bacterial strain was isolated from the rhizospheric soil of *Arachis hypogea*. Soil samples collected were incubated in 50 ml of LB medium for several hours. Using a sterile inoculation loop, inoculum was streaked onto the eosin methylene blue agar (EMB); based on the colony morphology and biochemical characteristics, it was identified as *E. cloacae*. The isolated organism was inoculated into 50 ml LB medium. After incubation, 0.5 ml of the supernatant was inoculated into 50 ml 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), and magnesium sulfate (1 g/l). After 2 days of cultivation at 37 °C, 0.5 ml of the culture broth was diluted with 50 ml of the same minimal medium (100-fold dilution) containing 2% glucose. These procedures were repeated three times in order to stimulate an enrichment culture. The enriched culture was used for PHA production. This strain was stored in minimal media with 25% glycerol solution at −70 °C for further use.

16s rRNA Gene Analysis

DNA Extraction

The isolated culture was subjected for the chromosomal DNA isolation. The chromosomal DNA was isolated using a slight modification of the method reported by Pitcher et al. [11]. Small amount of biomass from the minimal media agar plates were mixed and gently homogenized in 1.5-ml tubes containing 100 μl of TE buffer (pH 8.0) supplemented with 50 mg/ml lysozyme (Sigma, Ltd., Poole, Dorset, UK). The resulting solutions were incubated at 37 °C overnight, and 500 μl of a guanidine-sarcosyl solution (guanidine thiocyanate 60 g, 0.5 mM EDTA 20 ml, and deionized water 20 ml) was added to each preparation. The aqueous layers were separated by centrifugation and extracted with chloroform isoamyl alcohol (25:1, vol/vol). The chromosomal DNA was precipitated with 0.54 volume of isopropanol, washed in 70% (vol/vol) ethanol, and dried under a vacuum. The DNA samples were redissolved in 90 μl of TE buffer (pH 8.0), and 10 ml of RNase A (10 mg/ml; Sigma) was added prior to incubation at 37 °C for 2 h. After the RNase treatment, the DNA samples were extracted with phenol and chloroform, precipitated by

adding three volumes of ethanol in the presence of 0.8 M lithium chloride, washed with 70% ethanol, dried, and redissolved in 30 μl of water.

Determination of 16S rDNA Sequence

Phylogenetic Analysis

A 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′) [12]. The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, USA) according to the manufacturer's instructions and sequenced using a BigDye™ 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. The nucleotide (NT) sequence similarities were calculated using the PHYLODRAW program.

Optimization of PHA Production

The inoculum was prepared in minimal media, and 1% of inoculum was inoculated into 1 l media containing potassium dihydrogen phosphate (3 g/l), disodium hydrogen phosphate (6 g/l), ammonium chloride (2 g/l), sodium chloride (5 g/l), and magnesium sulfate (1 g/l). This media contained either glucose or lactose as carbon source at various concentrations (2, 3, 4, 5, 6, 7, 8, and 9 g/l). The optimum conditions for PHA accumulation at various incubation time (20–76 h, at a time interval of 2 h), pH (4–12), temperature (20–60 °C), and limiting any nutrition were determined.

Extraction of PHA

A 48-h-old culture was centrifuged, and the pellet was washed with distilled water, and PHA was extracted by modifying the method of Law et al. [13]. The culture was centrifuged at 14,333×*g* for 25 min at 4 °C, washed with distilled water, and freeze dried. One gram of the freeze-dried cell powder was treated with a dispersion containing 15 ml of chloroform and 30% sodium hypochlorite solution. The mixture was incubated at 37 °C with 250 rpm agitation for 1 h and then centrifuged at 2,610×*g* for 15 min. 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/ethanol at the ratio of 1:9). Finally, the white precipitate obtained was dried and weighed.

Determination of PHA Content in Cells

The PHA percentage in cells was calculated as the ratio of weight of extracted PHA to the cell dry weight, from which the PHAs were extracted.

Analytical Methods

Cell growth was monitored by measuring the optical density of the culture broth at 660 nm. The glucose/lactose concentration was analyzed by the Anthrone method.

Identification of Polymers

GC

PHA was extracted from 48-h-old culture grown in either lactose- (8 g/l) or glucose-containing (8 g/l) medium and subjected for methanolysis. Two milligrams of PHA was heated with a solution consisting 1 ml chloroform, 0.85 ml methanol, and 0.15 ml conc. H_2SO_4 at 100 °C for 40 min. Deionized water was added, and the lower phase was subjected for GC analysis (GC clarus 500 Perkin Elmer). Detector with a 30 m×0.25 mm ID×1 μm df capillary column was used. Helium (1 ml/min) was used as carrier gas. The injector and detector were at 250 and 200 °C, respectively. The program was run for 36 min, ramp of 2 min up to 110 °C, 10 °C per minute rise up to 200 °C, and hold at 280 °C for 5 min. This work was carried out at the Indian Institute of Crop Processing Technology (IICPT), Tanjore, Tamil Nadu, 613 001, India.

Results and Discussion

E. cloacae SU-1 was isolated from rhizospheric soil of *A. hypogea* and identified by colony morphology in yeast extract manitol salt agar and EMB and confirmed by its 16s rRNA sequence. Based on the similarity of the 16S rRNA gene, the isolate was found to be analogous to *Enterobacter* sp BSRA2 (98%), *E. cloacae* strain CMG 3058 (98%), *E. cloacae* strain FR (98%), and *E. cloacae* strain Rs-35 (98%). The isolate was determined to belong to *E. cloacae* based on the results of 16S rRNA gene sequencing and was designated as *E. cloacae* SU-1. The *E. cloacae* SU-1 isolated in this study is first time that has been examined for PHA production.

E. cloacae SU-1 was found to accumulate more PHA (85% of dry weight) in lactose-containing (8 g/l) medium, and it was found to accumulate more PHA (7.25% of dry weight) in glucose-containing (8 g/l) medium (Fig. 1). The concentration of PHA accumulated in lactose-containing medium was higher than in glucose-containing medium.

PHA accumulation in both the glucose- and lactose-containing minimal medium was gradually increased as the incubation time increased to 48 h; after that, it was found to be reduced (Fig. 2). Highest accumulation of PHA in lactose- and glucose-containing medium was found to be at pH 7.5 (Fig. 3). The organism was found to accumulate more PHA in lactose- (87% of dry weight) than in glucose-containing medium (7.5% of dry weight).

Figure 4 shows the effect of temperature in PHA accumulation. *E. cloacae* SU-1 was found to accumulate 94% PHA/dry weight at 30 °C in lactose-containing (8 g/l) medium, and only 8% of PHA/dry weight was accumulated in glucose-containing medium at 30 °C (Fig. 4). Carbon source and its concentration play a major role in the accumulation of PHAs, while limiting any nutrient in the medium was found to reduce the PHA accumulation (Table 1). It revealed that these nutrient sources also influenced PHA accumulation in *E. cloacae* SU-1. Optimal growth and PHB accumulation in *Bacillus megaterium* occurred with 5% (*w*/*v*) date syrup or beet molasses supplemented with NH_4Cl [14]. Joshi and Jaysawal [15] found that nitrogen source influences the accumulation PHA in microorganisms isolated from sewage.

The organism was found to accumulate PHA in the early stationary phase in both glucose- and lactose-containing medium. It was found to be utilized by the organism when it reaches the decline phase (Figs. 5 and 6).

PHA was extracted from *E. cloacae* SU-1 grown in lactose- (8 g/l) and glucose-containing (8 g/l) minimal medium. The extracted PHA was methanolysed and subjected

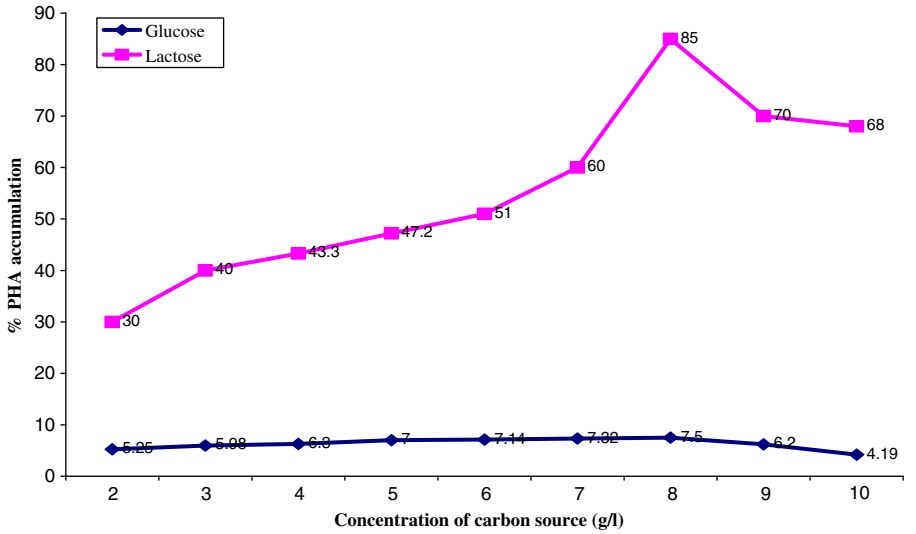

Fig. 1 PHA accumulation by *E. cloacae* SU-1 at various concentrations of glucose- and lactose-containing medium

for its monomer analysis using GC. The analysis showed the presence of the copolymers of 3-hydroxyoctanoate and 3-hydroxyhexanoate. The ratio of 3-hydroxyoctanoate (3HO): 3-hydroxyhexanoate (3HH) was found to be 6:1 in lactose-containing medium and 11:1 in glucose-containing minimal medium. PHAs are synthesized from fatty acids or other aliphatic carbon sources, and typically, the composition of the resulting PHA depends on the growth substrate used [16]. Medium chain length PHAs are also synthesized from carbohydrates, but

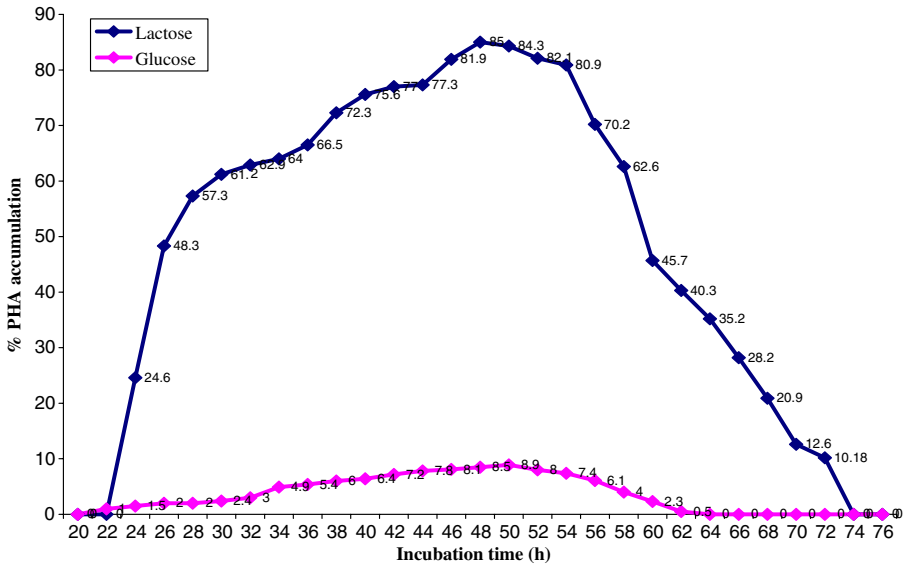

Fig. 2 PHA accumulation by *E. cloacae* SU-1 at various incubation times in glucose- and lactose-containing medium

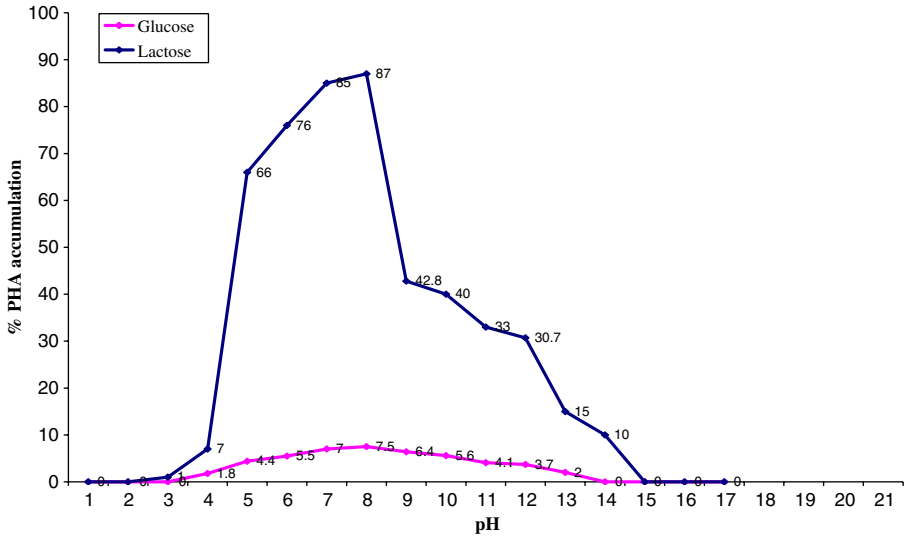

Fig. 3 PHA accumulation by *E. cloacae* SU-1 at various pH in glucose- and lactose-containing medium

the composition of these PHAs is not related to the carbon source [17]. The precursors for the synthesis of Mcl-PHA like PHO and PHH are *R*-3-hydroxyacyl-CoA in *Pseudomonas putida* [18]. *R*-3-Hydroxyacyl-CoA is synthesized from the de novo biosynthesis pathway of fatty acids or β-oxidation of fatty acids. In fatty acids de novo biosynthesis, the intermediates are

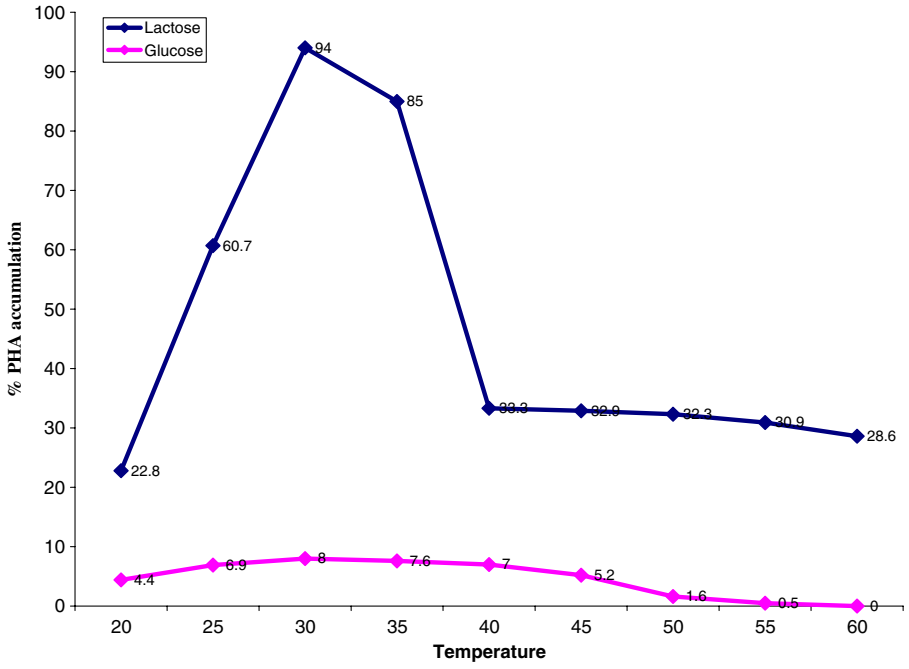

Fig. 4 PHA accumulation by *E. cloacae* SU-1 at various temperature in glucose- and lactose-containing medium

Table 1 PHA accumulation at nutrient limited conditions.

Source	PHA accumulation (%)	
	Lactose	Glucose
Potassium dihydrogen phosphate	25	5
Magnesium sulfate	50	6.4
Ammonium chloride	0	0
Disodium hydrogen phosphate	0	0

diverted towards PHA biosynthesis, where transacylase catalyzes the process. In β-oxidation of fatty acids, the oxidation of enoyl CoA to 3-hydroxyacyl-CoA was catalyzed by enoyl CoA hydratase. The 3-hydroxyacyl-CoA acts as substrate for the synthesis of Mcl-PHA [19, 20]. The vast majority of microbes synthesize either Scl-PHAs containing primarily 3-hydroxy butyrate units or Mcl-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers [21–23]. *E. cloacae* SU-1 was able to accumulate Mcl-PHA containing 3-hydroxyhexanoate (3HH) and 3-hydroxyoctanoate (3HO) in the presence of glucose and lactose. *Pseudomonas stutzeri* 1317 was also found to accumulate Mcl-PHA (HD, HO, HH, HDD, and HTD) from unrelated carbon sources, glucose [24]. The *E. cloacae* SU-1 was able to accumulate Mcl-PHA, which has not been reported in any literature so far. The strain seems to have a unique PHA synthase system that could work for the accumulation of Mcl-PHA.

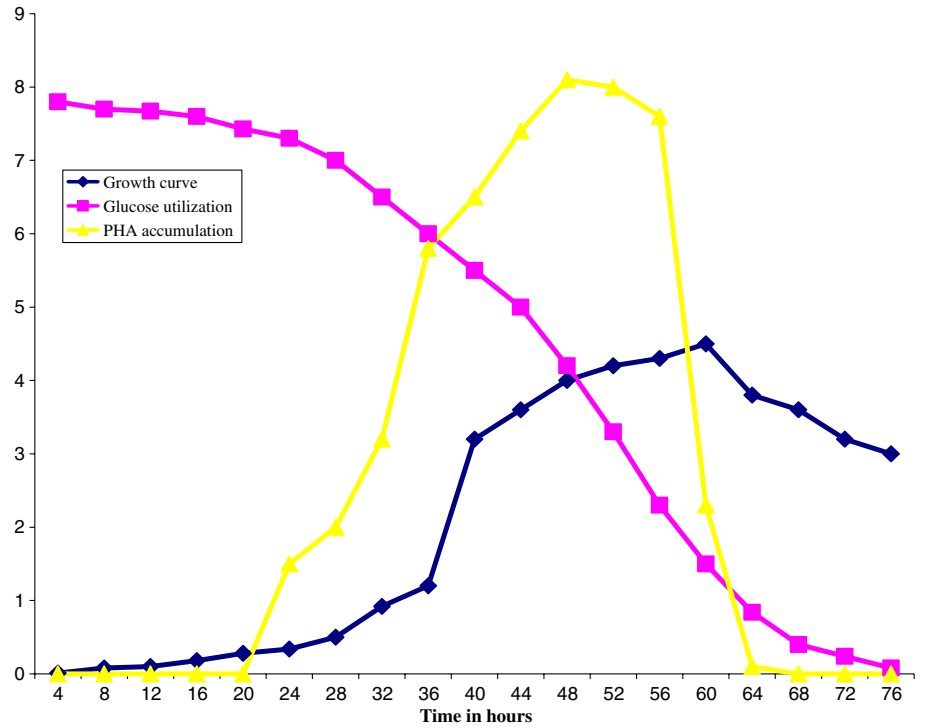

Fig. 5 PHA accumulation in glucose medium—growth vs PHA accumulation

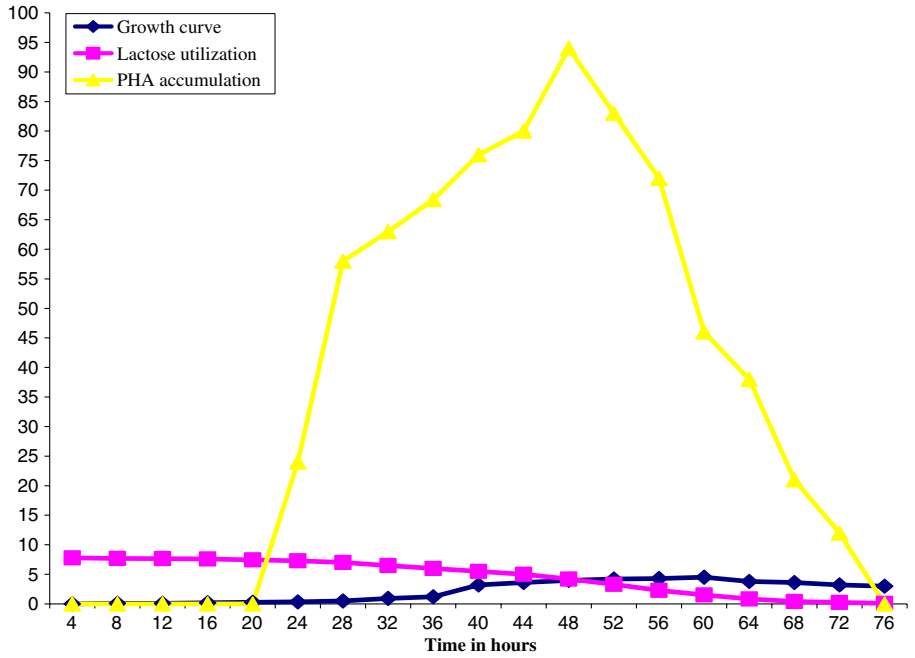

Fig. 6 PHA accumulation in lactose medium—growth vs PHA accumulation

Conclusion

E. cloacae SU-1 was able to accumulate higher concentration of PHA (94% of dry weight) when it was grown in minimal medium containing lactose (carbon source) 8 g/l at pH 7.5, temperature 30 °C for 48-h incubation. The organism was found to accumulate Mcl-PHA, 3-hydroxyoctanoate (3HO)/3-hydroxyhexanoate (3HH) in glucose- and lactose-containing medium, but the ratio of these monomers differed as 11:1 and 6:1, respectively.

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