5.1 INTRODUCTION

The production of enantiomerically pure chemicals has long been considered important in various sectors of the industry including food supplements, pharmaceuticals, cosmetics, fragrances, flavors, and other fine chemicals. In case of drugs such as thalidomide, the presence of an undesirable enantiomer leads to an increase in the dose required, and often also to adverse side effects (Roth, 2005). Many drugs are now synthesized using chiral synthons provided either by kinetic resolution of racemates, asymmetric synthesis or via the naturally occurring chiral pool (Sheldon, 1996). The replacement of conventional–chemical processes by sustainable biotechnological processes is one of the main current trends in white biotechnology and biocatalysis (Gavrilescu and Chisti, 2005). (R)-hydroxycarboxylic acids (R-HAs) can be widely used as chiral precursors for several reasons: (i) they contain at least two functional groups: a hydroxy group and a carboxy group; (ii) the functional groups can easily be modified chemically; and (iii) a second chiral center can be introduced. The reported compounds using R-HAs as chiral building blocks comprise the macrocyclic component of the antibiotic elaiophylin (Sutter and Seebach, 1983), the hydroxyacyl hydrazines in visconsin, a peptide antibiotic (Hiramoto et al., 1971), pharmaceuticals such as captopril and β-lactams (Ohashi and Hasegawa, 1992a,b) and fungicides such as norpyrenophorin and vermiculin (Seuring and Seebach, 1978). It has been reported that R-HAs can be obtained by hydrolysis of biotechnologically synthesized polyhydroxyalkanoates (PHAs) (Ren et al., 2010). PHAs are microbial polyesters, which are accumulated as a carbon and energy storage material under particular environmental conditions such as under nitrogen limitation.
1.5 Methods for PHA monomer production:

1.5.1 Chemical synthesis of R-HA

1.5.1.1 De novo synthesis

Introduction of the chiral center is the challenging step while producing enantiomerically pure R-HAs. Classical organic synthesis may include stereoselective oxidation through consecutive hydroxylation or through Brown’s asymmetric allylboration (Brown and Ramachandran, 1991). Enantiopure 3-hydroxyesters have been prepared chemically via enantioselective reduction using 3-keto esters as prochiral precursors (Noyori et al., 2004). General drawbacks of these reactions are the requirement of often expensive, chiral metal-complex catalysts, the contamination of end product with catalysts, and/or the high price of pure substrates. Vigorous reaction conditions such as high pressure, flammable reaction media or cryogenic conditions are often needed and the range of possible products is limited (Brown and Ramachandran, 1991; Ikunaka, 2003) Furthermore, the necessity to synthesize precursor molecules may complicate the synthetic procedure and may reduce the product yield (Nakahata et al., 1982; Wang et al., 1999).

1.5.1.2 Chemical degradation of PHA

Various enantiomerically pure R-HAs can be conveniently prepared by depolymerization of the biosynthesized PHA. A method for producing R-3hydroxybutyric acid and (R)-3-hydroxyvaleric acid (R-3HV) from PHB and poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate] (PHBV) by chemical degradation has been reported (Seebach et al., 1993). Lee et al., (1999) reported an efficient method for the preparation of R-3HB by acidic alcoholysis of PHB. De Roo et al., (2002) produced the chiral medium chain length (mcl) (R)-3-
hydroxycarboxylic acids via hydrolytic degradation of PHAs synthesized by *Pseudomonas putida*. PHAs were first degraded by acid methanolysis, and then obtained R-HA methyl esters were distilled into several fractions. Subsequently, the methyl esters were saponified to yield the corresponding (R)-3-hydroxycarboxylic acids.

1.5.2 Biosynthesis of HA

1.5.2.1 De novo biosynthesis

Hydroxyalkanoic acid can be produced by hydroxylation of fatty acids by organism. 3-Hydroxyvaleric acid has been synthesised by the hydroxylation of valeric acid in fermentation using *Candida rugosa* (Hasegawa et al., 1981), and a single enantiomer of 3HV (R-3HV) was similarly prepared using *P. putida, P. fluorescens, Arthrobacter oxydans* or *Arthrobacter crystallopietes* (Goodhue and Schaeffe, 1971). Several (R) and (S)-hydroxyalkanoic acids have been prepared by the reduction of corresponding fatty acid and recovered in the form of their methylester.

1.5.2.1a Enzymatic degradation of purified PHA in vitro

The ePhaZs partially degrade crystallized or denatured PHA, and the degradation products are typically R-HA monomers and dimmers.

1.5.2.1b Enzymatic degradation of PHA in vivo in wild-type bacteria

Another attractive approach to obtain R-HAs from PHAs is the *in vivo* depolymerization. The process utilizes intracellularly located PHA depolymerases for hydrolysis of PHAs. It has been efficiently accomplished with natural PHB-synthesizing bacteria to produce R-3HB with a yield of 96% (g R-3HB/g PHB) (Lee et al., 1999). Appropriate environmental conditions are crucial for this process. Lee et al. (1999) reported that, with *Alcaligenes latus* (reclassified as *Azohydrogenomonas*)
lata) (Xie and Yokota, 2005), lowering the environmental pH to 3-4 resulted in the highest activity of intracellular PHB depolymerase and blocked the reutilization of R-3HB by the cells. Under alkaline condition (pH 9-11) PHA depolymerase of P. putida give highest activity (Ruth et al., 2007). It has been demonstrated that, in many wild type bacteria, the in vivo biosynthesis and degradation of PHA simultaneously take place (Wang et al. 2013).

1.6 Regulation of PHA depolymerase synthesis

Synthesis of PHA depolymerases in bacteria is generally repressed if suitable soluble carbon sources such as glucose or organic acids are present. However, after exhaustion of the soluble nutrients synthesis of PHA depolymerases are derepressed in many strains (Jendrossek et al., 1993). At least in some bacteria dPHB depolymerase is expressed even in the absence of the polymer after cessation of growth. Therefore, an induction mechanism by the polymer itself is not necessary.

1.7 Potential applications of R-HAs

Since R-HAs contain a chiral center and two easily modified functional groups (–OH and –COOH), they are valuable synthons, i.e., they may serve as starting materials for the synthesis of fine chemicals such as antibiotics, vitamins, flavors, fragrances, and pheromones (Chiba and Nakai, 1985; Ohashi and Hasegawa, 1992a; Seebach et al., 2001). Various applications of R-HAs as synthons are listed below in Fig. 5.1 and table 1.
Figure 5.1: Various applications of R-HA (Ren et al., 2010)
### Table 5.1: Application of HAs as synthons (Ren et al., 2010)

<table>
<thead>
<tr>
<th>R-HAs</th>
<th>Potential synthon</th>
<th>Reference</th>
</tr>
</thead>
</table>
| (R)-3-hydroxyundec-10-enolate | Inhibitor of cholesterol synthesis, effects 3-hydroxy-3-methyl-glutaryl (HMG) CoA synthetase  
Chiang et al., (1989)             |
Katoh et al., (1994)              |
| (R)-3-hydroxyoctanoate        | Simvastatin (anti hypercholesterolemic, inhibitor of HMG-CoA reductase Viscosin | Lee and Lee, (2004); Morgan and Burk, (2005) 
Hiramoto et al., (1971)          |
| (R)-3-hydroxyhept-6-enoate    | Potent HMG CoA reductase inhibitor FR901512  
Rosuvastatin calcium, a HMG CoA reductase inhibitors  
α,β-disubstituted β-lactones  
Sphingofungin D (antifungal)  
Sphingofungin F (antifungal)  
Precursor of β-lactams for synthesis of carbacephems (class of antibiotics)  
Ebelactone A and B1 (β-lactone enzyme inhibitor)  
Bicycloheptenones  
Cyclosporine A derivatives (immunosuppressive)  
(S)-citronellol | Inoue and Nakada, (2007)       
Zlicar, (2007)                   
Wu and Sun, (2005)              
Mori and Otaka, (1994); Vanmiddlesworth et al., (1992) 
Kobayashi et al., (1997)         
Crocker and Miller, (1995)       
Paterson and Hulme, (1995)       
Marotta et al., (1994)           
Hirama et al., (1985)            |
| (R)-3-hydroxyheptanoate       | Anachelin (siderophore of *Anabaena cylindrica*)  
Keri et al., (2007)              |
1.7.1 Medical applications

R-3HB has been employed to treat traumatic injuries such as hemorrhagic shock, extensive burns, myocardial damage, and cerebral hypoxia, anoxia, and ischemia (Massieu et al., 2003; Tieu et al., 2003; Zou et al., 2009). It was also shown that 3HB oligomers provide energy and show good penetration and rapid diffusion in peripheral tissue; hence, they could be an energy substrate for injured patients (Tasaki et al., 1999). R-3HB could also serve as energy substrate in increasing cardiac efficiency and thus, prevents brain damage (Kashiwaya et al., 2000). There is also evidence that R-3HB can correct defects in mitochondrial energy generation in the heart (Katayama et al., 1994). Furthermore, R-3HB has been found to be able to reduce the death rate of the human neuronal cell model culture for Alzheimer’s and Parkinson’s diseases and to ameliorate the appearance of corneal epithelial erosion through suppression of apoptosis (Kashiwaya et al., 2000). R-3HB methyl ester was also found to dramatically improve the memory of mice (Zou et al., 2009).

1.7.2 Antimicrobial agents

Sandoval et al., (2005) reported that (R)-3-hydroxy-n-phenylalkanoic acid can effectively attack Listeria monocytogenes, which is a ubiquitous microorganism, and able to multiply at refrigeration temperatures and is resistant to both high temperature and low pH. R-3HB has been shown to exhibit some antimicrobial, insecticidal, and antiviral activities (Chen and Wu, 2005; Shiraki et al., 2006). Radivojevic et al., (2015) reported that R-3-hydroxyoctanoic acid monomers and its derivatives derived from a bacterial polyester PHA have antimicrobial activity and in vitro antiproliferative effect with mammalian cell lines.
Aim and scope of the chapter

Since R-Hydroxyalkanoic (R-HAs) acids contain a chiral center and two easily modified functional groups (–OH and –COOH), they are valuable synthons, i.e., this serve as starting materials for the synthesis of fine chemicals.

Development of method for the synthesis of more complex and useful chiral compounds by enzymatic approach i.e. in vivo depolymerization of synthesized PHA will help to overcome general drawbacks of complex, monotonous and often expensive chemical process. We have already carried out value added production of PHA using agro-industrial wastes by P. aeruginosa. Synthesis of R-HAs by in vivo depolymerization of synthesized PHA by P. aeruginosa using glycerol has been investigated in the present study.
5.2 MATERIALS AND METHODS

5.2.1 Bacterial strain

P. aeruginosa accumulate about 42% of PHA per dry cell mass at 30 °C, pH 8 after 72 h was used for hydroxyalkanoic acid production.

5.2.2 Accumulation of PHA in P. aeruginosa

P. aeruginosa was allowed to accumulate PHA upto 72 h in BHM medium containing 1% waste glycerol. After the 72 h of growth cells were harvested by centrifugation at room temperature in sterile oak ridge tubes at 8000 rpm for 10 min and cells were used for in vivo depolymerization of accumulated PHA.

5.2.3 In vivo depolymerization of PHA

In vivo depolymerization of PHA requires the biomass with PHA accumulated to be harvested from fermentation broth first, and then PHA degradation is triggered by modifying the pH or temperature (Ren et al., 2005) In vivo depolymerization of PHA for the production of hydroxyalkanoic acid was carried out with P. aeruginosa where organism is allowed to depolymerise the PHA, which has accumulated during its growth on production medium. Hydroxyalkanoic acid production during in vivo depolymerization of PHA was measured in terms of PHA depolymerase activity. PHA depolymerase activity was assayed by the determination of NaOH consumption rates necessary to keep the pH of buffered cell suspension at constant indicated pH (Gebauer and Jendrossek, 2006) because of the release of R-HAs from reserve PHA. Equation for determination of PHA depolymerase activity is given below. pH-stat apparatus equipped with Tiamo 1.1 software was used (Titrando 902, Metrohm India Limited). Many factors like pH of the medium, temperature and concentration of cells affects the in vivo depolymerization. Considering this fact, optimization of various
parameters for \textit{in vivo} depolymerization of PHA for hydroxyalkanoic acid production was carried out.

\[
\text{PHA depolymerase activity} = \frac{\text{Molarity of NaOH} \times \text{Volume of NaOH} \times 1000}{\text{Incubation Time} \times \text{Volume of Sample}}
\]

5.2.3.1 Optimization of pH

In the majority of reaction kinetics, the chemical reactions are dependent on the consumption of \( \text{H}_3\text{O}^+ \) or \( \text{OH}^- \) ions. The speed of formation depends on operating conditions, in particular the pH of the reactive media. The effect of pH on the production of hydroxyalkanoic acid monomers was analysed at different pH 7, 8, 9, 10 and 11 using 50 mM potassium phosphate buffer. One gram of cells (wet cell weight) inoculated in 60 ml of potassium phosphate buffer and then buffer was titrated to achieve respective pH. Decrease in pH due to generation of hydroxyalkanoic acid monomers or oligomers were recorded after every 15 min. After 4 h of incubation the suspension was titrated with 0.1 N NaOH to respective pH. The cells were harvested by centrifugation at 8000 rpm for 10 min and supernatant was collected. The supernatant was analysed for monomers or oligomers production.

5.2.3.2 Optimization of temperature

Effect of different incubation temperature was also analyzed for maximum hydroxyalkanoic acid monomers or oligomers production. For that reaction was carried out in phosphate buffer (pH 10) at 30 °C and 37 °C. Effect of temperature on monomer or oligomers production was analyzed after 4 h of incubation.
5.2.3.3 Optimization of cell concentration

Effect of different cell concentration i.e 0.5 g, 1.0 g and 1.5 g (wet cell weight) on hydroxyalkanoic acid production was determined by inoculating respective cell concentration in 50 mM potassium phosphate buffer of pH-10 at 37 °C.

5.2.4 Isolation of monomers and oligomers

The NaOH consumption rate was measured after in vivo depolymerization of PHA and the reaction mixture was centrifuged at 8000 rpm for 10 min. The supernatant containing the reaction product was concentrated by allowing buffer to evaporate using rotary evaporator (Heidolph, Germany) and then it was used for product determination. Alternatively, the reaction mixture was acidified with HCl to pH 3 to stop any enzymatic activity and to protonate 3HB monomers or oligomers (Gebauer and Jendrossek, 2006). After centrifugation for 30 min, the supernatant was extracted with ethylacetate. 3HB monomers or oligomers solubilised in the organic phase were isolated by evaporation of solvent.

5.2.5 Detection of PHA monomers and oligomers

5.2.5.1a Preparation of hydroxyalkanoic acid methylesters and thin layer chromatography

Hydroxyalkanoic acid methylesters were obtained via acid catalyzed hydrolysis of product. Methanolysis was carried out in the presence of 15% (v/v) H₂SO₄ (Roo et al., 2002; Brandl et al., 1988). The tubes were allowed to cool and methylesters were extracted with equal volume of hexane. Formation of hydroxyalkanoic acid methylesters was confirmed by TLC using diethyl ether: acetic acid (90:10) as a mobile phase (Ichihara et al., 2010). Detection of samples was carried out with the iodine vapors.
5.2.5.1b Detection of methylester by GC

The prepared methylesters were analyzed by Gas chromatography (Perkin-Elmer Auto system XL, USA). Sample was analyzed at 200 °C oven temperature using nitrogen as a carrier gas with flame ionization detector.

5.2.5.2a Derivatization of monomers and oligomers

The method used for the derivatization of PHB hydrolysis products is based on a procedure described previously by Durst et al. (1975) for fatty acids. One hundred microliters of sample containing 1 mM to a maximal 5 mM 3HB or 3HB oligomers was alkalized by the addition of 100 µl triethylamine (0.1 M in acetone). The solvents were evaporated under nitrogen gas. One hundred fifty microliters of bromophenacyl bromide (BPB) (10 mM in acetonitrile) and 150 µl crown ether (decyl-18-crown-6) (2 mM in acetonitrile) were added, and the reaction mixture was incubated with a sealed lid at 80 °C for 90 min. The reaction tubes were vortexed several times during the incubation and were cooled to room temperature at the end of the incubation time.

5.2.5.2b Detection of PHA monomers and oligomers by reverse-phase C\textsubscript{18} HPLC

Twenty microliters of the derivatization mixture was loaded onto a reverse-phase C\textsubscript{18} HPLC column (5 µm, 4.6 × 150 mm) (Eclipse XDB-C\textsubscript{18}; Agilent). Samples were eluted at a flow rate of 0.8 ml/min. Buffers used were 0.01 M ammonium formate with 2% methanol, pH 4 (solution A), and pure methanol (solution B). Elution conditions were as follows: gradient of 60% solution A–40% solution B for 27 min then 100% solution B at 28 min, isocratic run at 100% solution B for 5 min (33 min), gradient to starting conditions (60% solution A–40% solution B) within 1 min (34 min), and an isocratic run at 60% solution A–40% solution B for 5 min (39 min). The absorbance of bromophenacyl bromide derivates was detected at 254 nm (Gebauer
and Jendrossek, 2006). BPB was used as a reference. A peak at 16.1 min corresponded to unreacted BPB.

5.3 RESULTS AND DISCUSSION

5.3.1 In vivo depolymerization of PHA

The intracellular mcl-PHA depolymerase activity was measured for the first time as an auto hydrolysis of mcl-PHA granules from a strain of *P. oleovorans* (Foster et al., 1994). An in vivo method for the production of chiral hydroxyalkanoic acid monomers from mcl-PHA has been reported by Ren et al., (2005) using *P. putida* strain. Similarly Ruth et al., (2007) also reported that R-3-Hydroxycarboxylic acids from *P. putida* GPo1 were obtained when condition were provided to promote in vivo depolymerization of intracellular.

5.3.1.1 Influence of pH on in vivo depolymerization of PHA

The effect of pH on the production of hydroxyalkanoic acid monomers was studied at different pH 7, 8, 9, 10 and 11 using 50 mM potasium phosphate buffer. Previous reports have indicated that controlling pH of reaction mixture can shift metabolic pathway to enhance in vivo depolymerization of PHA and thus favor R-HAs production (Lee et al., 1999; Ren et al., 2005). Potassium phosphate buffer is used because it lacks the carbon source and in the starvation condition cell degrades accumulated PHA and produces 3HAs using intracellular PHA depolymerase. Experiment was carried out with pH stat. Use of pH stat (Titrando 902, Metrohm India Limited) for in vivo depolymerization study has been suggested in previous reports (Gebauer and Jendrossek, 2006; Wang et al., 2007). Decrease in pH was recorded after every 15 min and time profile for decrease in pH is shown in Fig. 5.2. Decrease in pH of reaction mixture was attributed to the generation of hydroxyalkanoic acid monomers or oligomers during in vivo depolymerization. PHA
depolymerase activity was measured by the determination of NaOH consumption. Maximum decrease in pH and maximum PHA depolymerase activity was observed at pH 10 (Fig. 5.2, 5.3). At pH 7, no apparent decrease in pH and PHA depolymerase activity was observed. *P. aeruginosa* showed the maximum PHA depolymerase activity at pH 10 (0.0714 U/ml) i.e under alkaline condition after 4 h.

**Figure 5.2: Time profile of 3-HA production at different static pH**

**Figure 5.3: Effect of static pH on *in vivo* PHA depolymerase activity**

Wang et al., (2007) suspended *P. putida* cells containing PHA in phosphate buffer at different pH (pH 9-11), the degradation of PHA and thus, the release of R-HA monomers were studied. Gangoiti et al., (2010) and Wang et al., (2007) reported the maximum depolymerase activity at pH 9.5 and 9.2, respectively. The reports state that
the *in vivo* hydrolysis of PHA and secretion of 3-hydroxyalkanoic acids by *Pseudomonas* sp. can be efficient at a pH level of 10 to 11 (Ren et al., 2005; Wang et al., 2007).

### 5.3.1.2 Influence of temperature on *in vivo* depolymerization of PHA

Cells of *P. aerugenosa* suspended in distilled water to achieve the concentration of 1g (wet cell weight), were inoculated in 50 mM potassium phosphate buffer (pH 10) and temperature of reaction vessel was maintained at 30 °C and 37 °C separately. The time profile of R3-HA production is shown in Fig. 5.4. Maximum decrease in pH was observed at 37 °C (Fig. 5.4). Higher production of hydroxyalkanoic acid was observed at temperature 37 °C with maximum enzyme activity of 0.0724 U/ml (Fig. 5.5). *Alcaligenes latus* is also reported for the maximum R3HA production at 37 °C by Sang et al., (1999). *Pseudomonas oleovorans* is also reported to depolymerize accumulated PHA *in vivo* at 37 °C to produce different monomers like R3HH, R3HN and R3HPV (Sang et al., 1999).

![Figure 5.4: Time profile of 3-HA production at different temperature](image)

*Figure 5.4: Time profile of 3-HA production at different temperature*
5.3.1.3 Influence of cell concentration for \textit{in vivo} depolymerization of PHA

For optimization of cell concentration for \textit{in vivo} PHA depolymerase activity, different concentration of cells such as 0.5 g, 1.0 g and 1.5 g (wet cell weight) were inoculated in 50 mM phosphate buffer at 37 °C pH 10. Activity was measured after 4 h. Maximum change in pH with time (Fig. 5.6) and maximum depolymerase activity of 0.0730 U/ml (Fig. 5.7) was obtained with cell concentration of 1g. Gao et al., (2002) reported that recombinant \textit{E.coli} DH5α containing phaA and phaB genes from \textit{R. eutropha} and buk and ptb genes from \textit{C. acetobutylicum} gave 3-HA production with 5.5 g/l.
Synthesis of R-HAs by in vivo depolymerization

5.3.2 Detection of PHA monomers or oligomers

5.3.2.1 Thin layer chromatography and gas chromatography

Preliminary identification of HAs obtained under optimized condition was carried out by preparing methylesters. Methylesters were prepared by acidic methanolysis which
were loaded on TLC plates and the hydroxyalkanoic acid methylester was appeared as different spots on TLC plate, observed in Fig. 5.8.

**Figure 5.8:** Thin layer chromatography of hydroxyalkanoic acid methylester

**Figure 5.9:** Chromatogram of hydroxyalkanoic acid produced in optimized condition

Products obtained in optimized condition were converted to the simplest convenient volatile derivative, methylesters and analyzed by Perkin Elmer Gas chromatograph. Gas chromatogram of product confirms the presence of few hydroxyalkanoic acid
methylesters ranging from C₄ to > C₈ (Fig. 5.9). Similarly hydroxyalkanoic acid methylesters were assayed by gas chromatography by Timm and Steinbuchel, (1990). Ruth et al., (2010) also reported the detection of hydroxyalkanoic acid methylesters by GC analysis.

5.3.2.2 Detection of PHA monomers or oligomers by reverse-phase C₁₈ HPLC

Production of hydroxyalkanoic acid was carried out at pH 10, with 1.0 g cell concentration at 37 °C i.e. at optimized condition. Hydrolysis product after in vivo depolymerization of PHA was isolated as mentioned in 5.2.4. As 3HA monomers and oligomers have only poor absorption at 210 nm direct UV detection of the monomers and oligomers is therefore possible only for high concentrations of products (Gebauer and Jendrossek, 2006). Alkylation of hydroxyalkanoic acid with BPB as a chromophore helps in detection and separation by HPLC.

![HPLC chromatogram of bromophenacyl bromide](image)

**Figure 5.10: HPLC chromatogram of bromophenacyl bromide**
HPLC chromatogram of a bromophenacyl bromide is shown in Fig. 5.10. A peak at 14.38 min corresponds to bromophenacyl bromide (Fig. 5.10). Hydroxyalkanoic monomers from two to more than eight monomer units produced during \textit{in vivo} depolymerization were reacted with bromophenacyl bromide and those bromophenacyl derivates separated on a reverse phase C$_{18}$ column shown in Fig. 5.11. A peak at 11.08 min corresponds to 3-hydroxybutyrate, 14.35 min represents BPB and 22.62 min shows the presence of 3-hydroxyoctanoate (Fig. 5.11). Results obtained during HPLC are similar to Gebauer and Jendrossek (2006). Similarly Ruth et al., (2007) also used HPLC methods for the analysis of monomers and oligomers. Wang et al., (2007) reported that under alkaline conditions, PHAs containing R-3-hydroxyoctanoic acid (R-3HO) and R-3HHx were degraded with a good efficiency in 9 h.

**CONCLUSION:**

PHA accumulated in \textit{P. aeruginosa} after 72 h was depolymerised by the action of PHA depolymerase to liberate hydroxyalkanoic acid in potassium phosphate buffer of 10 pH with a cell concentration of 1g (wet cell weight) at 37 °C. Various (R)-3-
hydroxycarboxylic acids can be produced via *in vivo* depolymerization of previously accumulated PHA in *P. aeruginosa* in controlled buffer system using pH-stat. Derivatization of produced hydroxyalkanoic acid with bromophenacyl bromide made detection of products R-3HB and R-3HO easy by reverse phase C$_{18}$ HPLC.