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In vivo and in vitro depolymerizations of intracellular medium-chain-length poly-3-hydroxyalkanoates produced by Pseudomonas putida Bet001

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ABSTRACT
In vivo and in vitro depolymerizations of intracellular medium-chain-length poly-3-hydroxyalkanoates (mcl-PHA) in Pseudomonas putida Bet001 grown on lauric acid was studied. Both processes were studied under optimum conditions for mcl-PHA depolymerization viz. 0.2 M Tris-HCl buffer, pH 9, ionic strength (I) = 0.2 M at 30°C. For in vitro depolymerization studies, cell-free system was obtained from lysing bacterial cells suspension by ultrasonication at optimum conditions (frequency 37 kHz, 30% of power output, <25°C for 120 min). The comparison between in vivo and in vitro depolymerizations of intracellular mcl-PHA was made. In vitro depolymerization showed lower depolymerization rate but higher yield compared to in vivo depolymerization. The monomer liberation rate reflected the mol% distribution of the initial polymer subunit composition, and the resulting direct individual products of depolymerization were identical for both in vivo and in vitro processes. It points to exo-type reaction for both processes, and potential biological route to chiral molecules.

KEYWORDS
Cell-free biology; depolymerization; in vitro; in vivo; polyhydroxyalkanoates

Introduction
Biodegradable polymers are defined as degradable materials in which the degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide (in the case of aerobic degradation) and/or methane (in the case of anaerobic degradation), and a new cell biomass. Polyhydroxyalkanoates (PHAs) are one such example because they can undergo biodegradation at various environmental conditions. It is a family of biopolymers with diverse structures, and synthesized by microorganisms as reserve granules. It is considered as an environmental-friendly and sustainable alternative, especially in the face of rapid consumption of non-renewable fossil-based polymers. The potential of PHA is not limited to the versatility and flexibility of the neat polymer but also its biodegradation products such as monomers, dimers, and oligomers due to the chiral purity which provide a new route to the synthesis of platform chemicals.

More than 150 different types of hydroxyalkanoates have been identified as monomer constituents resulting in exceptionally diverse structures of PHA. In PHA, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. Each monomer contains the chiral carbon with (R)-stereochemical configuration. R-3-hydroxyalkanoic acids (R3HAs) produced by the hydrolysis of PHAs can be used as starting materials in fine chemicals, pharmaceutical, and medical industries. Nowadays, PHA monomers can be produced by depolymerizing the biosynthesized, accumulated PHA using different ways. Methods for producing R3HAs by chemical digestion have been reported. However, large amounts of organic solvents are needed for the process in addition to low production efficiency and multi-step requirements thus making the biological route for R3HAs production to be potentially more viable.

Intracellular PHA, which is accumulated as storage granule in bacterial cell can be degraded by several hydrolyses, and the degradation products are used as carbon and energy source by the bacteria during period of starvation. The main enzyme that catalyzes the depolymerization of accumulated PHAs within bacterial cell are called intracellular PHA (iPHA) depolymerases. Researches on the biodegradation of PHA should clearly distinguish between intracellular- and extracellular PHA depolymerizations. Intracellular depolymerization is the hydrolysis of an endogenous carbon reservoir by the accumulating bacteria themselves, whereas extracellular depolymerization is the utilization of an exogenous carbon source not necessarily by the accumulating microorganisms alone but also other organisms. Intracellular native PHA (nPHA) granules exhibit amorphous, rubbery state. nPHA consists of highly mobile chains of the carbon backbone giving it a disordered conformation in addition to being covered by a surface layer of protein and phospholipid. Upon extraction from the cell, or after cell lysis or death, the surface layer of nPHA granules is rapidly damaged or lost, and the PHA chain tends to adopt an ordered helical conformation and develop a crystalline phase known as denatured PHA.
Intracellular PHA depolymerization could occur as in vivo and in vitro processes. In vivo depolymerization of intracellular PHA occurs on the PHA granule where iPHA depolymerases is also located, and the activity is associated with the protein complex encompassing the nPHA inclusion bodies inside the bacterial cells. Degradation takes place without isolation and purification of the iPHA depolymerases enzyme and nPHA as a substrate.\(^5\)\(^,\)\(^10\)\(^,\)\(^19\) However, in vitro depolymerization of iPHA occurs when the polymer granules are isolated from bacterial cell with their protein coat intact without losing their amorphous and elastomeric nature characteristics, and the iPHA depolymerase remains active on the isolated PHA inclusion bodies.\(^15\)\(^,\)\(^19\)\(^–\)\(^21\)

Recently, various enantiomerically pure R3HAs have been produced by in vivo depolymerization of iPHA since in vitro depolymerization of iPHA is a tedious method with significant difficulties in nPHA isolation, and purification of intracellular depolymerase enzyme without losing nPHA amorphous characteristic and enzyme activity. Isolated nPHA granules and iPHA depolymerase enzyme are extremely sensitive to chemical and physical stresses.\(^3\)\(^,\)\(^22\) Despite the limitations, it is possible to obtain higher depolymerization rate for in vitro depolymerization of intracellular nPHA compared to in vivo as in short-chain-length PHA.\(^20\) However, equivalent process has not been well-studied in the case of medium-chain-length (mcl) nPHA. In this study, in vitro depolymerization of intracellular mcl-PHA was investigated using cell-free biology system. Cell-free biology involves the activation of complex biological processes without using living cells.\(^23\)\(^,\)\(^24\) The cells are opened up (lysed) and unpurified portions are used. In cell-free technologies, source cells are grown, harvested, and then lysed. The lysate can be used directly or centrifuged to remove suspended solids and further processed, if needed.\(^24\) For the purpose of current investigation, crude preparation of lysed cell components was used without further purification nor pretreatment. By exploiting PHA machinery of Pseudomonas putida Bet001 as a model system for mcl-PHA depolymerization process, a comparison between in vivo and in vitro depolymerizations of intracellular mcl-PHA was also made.

**Materials and methods**

**Microorganism and biosynthesis of mcl-PHA**

_Pseudomonas putida_ Bet001 isolated from palm oil mill effluent was used in this study.\(^25\) Bacterial cells from glycerol stock was reconstituted into nutrient broth (NB) for 24 h prior to culturing into nutrient rich (NR) medium. Bacterial cells solution (1 ml) from NB was aseptically inoculated into NR medium containing (g L\(^{-1}\)): yeast extract 10.0 (Bacto\(^\text{TM}\), France), nutrient broth 13.0 (OXOID, England), and (NH\(_4\))\(_2\)SO\(_4\) 5.0 (Sigma-Aldrich, Germany) in 100 ml per each 250 ml shake-flasks. The culture was incubated in a shaking incubator (Daihan LabTech\(^\text{®}\), Korea) at 25 ± 1°C, 200 rpm for 24 h. Subsequently, the harvested culture broth was centrifuged at 4°C, 3,578\(\times\)g for 5 min and the biomass recovered was washed twice with saline solution (0.9% w/v). The cell suspension in sterile distilled water (1 g L\(^{-1}\)) was inoculated aseptically into PHA production medium (100 ml per each 250 ml shake-flasks). The autoclaved medium contained (g L\(^{-1}\)): NaNH\(_4\)HPO\(_4\), 4H\(_2\)O 3.5, KH\(_2\)PO\(_4\) 3.7, K\(_2\)HPO\(_4\) 5.7. It was supplied with lauric acid (C\(_{12}\)) (0.56 g in 100 ml medium) as a sole carbon and energy substrate but with limited nitrogen source to obtain C/N ratio at 20 (mol:mol). Separate sterile solutions of 0.1 M MgSO\(_4\) \(\cdot\) 7H\(_2\)O (1.0% v/v) and 0.1% v/v trace elements (MT) solution containing (g L\(^{-1}\)): CaCl\(_2\) \(\cdot\) 2H\(_2\)O 1.47, CoCl\(_2\) \(\cdot\) 6H\(_2\)O 2.38, CuCl\(_2\) \(\cdot\) 2H\(_2\)O 0.17, FeSO\(_4\) \(\cdot\) 7H\(_2\)O 2.78, MnCl\(_2\) \(\cdot\) 4H\(_2\)O 1.98, and ZnSO\(_4\) \(\cdot\) 7H\(_2\)O 0.29 dissolved in 1 M HCl were added aseptically to autoclaved liquid medium at room temperature. Following inoculation, the flasks were incubated under aerobic conditions at 25°C and 200 rpm for 48 h. After cultivation, the harvested culture broth was centrifuged at 4°C, 3,578 \(\times\) g for 5 min and the biomass recovered was washed twice with saline solution (0.9%, w/v) and n-hexane to remove residual fatty acids. The bacterial mass was dried in an oven at 70°C until constant weight. The dried biomass was used to determine the mcl-PHA content and monomer composition using gas chromatography (GC). The mcl-PHA comprised of 3-hydroxyhexanoate (C\(_6\)) (3HHx) at 6.12 mol%, 3-hydroxyoctanoate (C\(_8\)) (3HO) at 32.78 mol%, and 3-hydroxydecanoate (C\(_{10}\)) (3HD) at 3.27 mol%, and 3-hydroxydodecanoate (C\(_{12}\)) (3HDD) at 21.04 ± 3.15 mol% (Figure 1).

**Sample preparation**

Samples for in vitro depolymerization of intracellular mcl-PHA were prepared by lysing the bacterial cells containing intracellular mcl-PHA in microprocessor-controlled ultrasonic bath (Elmasonic P 30H, Germany) sonicator. The cells suspension was sonicated at 37 kHz frequency, 30% of power output,\(^26\) with temperature controlled <25°C for 120 min (except stated otherwise). At the indicated ultrasonic settings,

**Figure 1.** GC chromatogram of monomers and its proportion produced in the _Pseudomonas putida_ Bet001 using lauric acid as a sole carbon source. (3-hydroxyhexanoate (C\(_6\)) (3HHx); 3-hydroxyoctanoate (C\(_8\)) (3HO); 3-hydroxydecanoate (C\(_{10}\)) (3HD); 3-hydroxydodecanoate (C\(_{12}\)) (3HDD); methyl benzoate (MB) as internal standard.)
Effects of different sonication times on in vitro depolymerization of intracellular mcl-PHA

Different sonication times for the preparation of crude samples were used in in vitro depolymerization studies. The crude sample preparations exposed to different sonication times (90, 120, and 150 min) were collected and added to a buffer solution 0.2 M Tris-HCl (final volume 100 ml), pH 9, and ionic strength ionic strength $I = 0.2$ M at 30°C for 24 h. The most suitable sonication time was used for further experiments as preparation sample.

In vivo and in vitro depolymerizations of intracellular mcl-PHA

In vivo

Bacterial cells harvested from PHA production medium were recovered by centrifugation, washed, and resuspended into PHA depolymerization medium (0.2 M Tris-HCl buffer, pH 9, $I = 0.2$ M at 30°C) from the previous in vivo depolymerization of intracellular mcl-PHA studies. The cells were statically incubated for 48 h and time profiling was performed. The cells in depolymerization medium were harvested at 12-h intervals. The bacterial cells were centrifuged and oven dried after in vivo depolymerization to analyze the total biomass amount, mcl-PHA content, and monomer composition. The supernatant was collected to profile exogenous depolymerization products of interest.

In vitro

Following cultivation, the harvested culture broth was centrifuged, and the biomass recovered was washed twice with saline solution (0.9%, w/v). Then the pellet was resuspended in approximately 10 ml of 0.2 M Tris-HCl buffer, pH 9, $I = 0.2$ M prior to sonication. After sonication, 0.2 M Tris-HCl buffer pH 9, $I = 0.2$ M was added to the lysed cells mixture (5.73 g L$^{-1}$) to a final volume of 100 ml for in vitro depolymerization process (in 250 ml shake-flasks). The medium, i.e., 0.2 M Tris-HCl buffer, pH 9, $I = 0.2$ M at 30°C, was adopted from the previous in vivo depolymerization of intracellular mcl-PHA studies. The lysate preparations were statically kept for 48 h and time profiling was performed. The suspension samples were harvested at 12-h intervals. They were centrifuged and oven dried after in vitro depolymerization to analyze the total pellet fraction weight, mcl-PHA content, and monomer composition. The supernatant was collected to profile exogenous depolymerization products of interest.

Enzymatic depolymerization activity

One unit (1 U) of intracellular enzymatic depolymerization activity is defined as the total enzyme action that liberates 1 μmol per min the monomers available within the mcl-PHA. Bacterial cells containing mcl-PHA exposed to thermal treatment at 70°C for 10 min were used as control for in vivo and in vitro depolymerization experiments.

Biomass estimation

Bacterial growth was rapidly estimated by taking the optical density (OD) readings of the cell culture. The OD was determined at a wavelength of 600 nm (OD$_{600}$) using Jasco V-630 UV/VIS spectrophotometer (Jasco, Japan). A standard calibration for $P$. putida Bet001 biomass against OD$_{600}$ was prepared by serial dilution of known amount of cell biomass. 1 unit OD$_{600}$ is equivalent to 0.37 g L$^{-1}$ of cell dry weight.

mcl-PHA extraction

mcl-PHA extraction was performed by resuspending the dried cells in dichloromethane (DCM) (Merck, USA) and then refluxed for 4 h at 60 ± 5°C. Buchner filter funnel with sintered glass was used to filter out non-PHA biomass component. Extracted mcl-PHA in the filtrate was then concentrated under vacuum in a rotary evaporator at 60°C and 70 rpm until about 1/6 of total volume. A beaker containing excess cold methanol (Merck, USA) was used to precipitate mcl-PHA, in the volume ratio of 1:4. Purification steps were repeated three times by redissolving the product in DCM followed by reprecipitation in cold methanol. mcl-PHA content (% of total biomass) was determined based on its gravimetric analysis against dried total biomass as reported previously.

Analyses

GC analysis

mcl-PHA concentration and monomer composition in bacterial cells were determined using gas chromatography (GC) (Trace GC Ultra, Thermo Scientific, Italy) equipped with a flame ionization detector and a fused silica capillary column (30 m length × 0.32 mm internal diameter, 0.25 μm film) (Supelco SPBTM-1, USA). Approximately 10–20 mg of dry cells were subjected to methanolysis in the presence of chloroform with methanol and sulfuric acid [85%-15% (v/v)]. The reaction mixture was incubated at 100°C for 140 min. The organic layer containing the reaction products was separated, dried over Na$_2$SO$_4$, and analyzed by GC. Benzoic acid methyl ester was used as the internal standard. 1.0 μl of methanolyzed PHA sample was injected by splitless injection. Helium was used as the carrier gas at a flow rate of 2 ml min$^{-1}$. The column oven initial temperature was programmed at 50°C, then ramped up at a rate of 10°C min$^{-1}$ to 280°C, and held at this temperature for 2 min. The temperatures of injector and detector were set at 200 and 280°C, respectively. The PHA standard monomers of methyl hydroxyalkanoates (Larodan, Sweden and Aldrich, Germany) were used as representative references for peak retention time and standard calibration.
for each monomer concentration. mcl-PHA content (%) was obtained by summing up all individual monomers present and given as mcl-PHA weight (mg) per total dried biomass (mg).

The supernatant from in vivo and in vitro depolymerizations of intracellular mcl-PHA was collected after centrifugation at 4°C, 3,578 × g for 5 min. It was acidified using concentrated HCl to pH 1.0. The acidified supernatant was filtered (0.22 µm) to remove residual cell debris. The supernatant was then dried before extraction in DCM and subjected to silylation for detection and quantification of exogenous depolymerization products of interest (i.e., monomers or possibly oligomers) by gas chromatography–mass spectrometry (GC–MS). GC–MS analysis was recorded on Shimadzu GC (model 2010) coupled to Shimadzu QP 2010 Ultra MS (Shimadzu, Japan) operated in electron impact ionization mode at 70 eV with RTX-5 column (Restek Corporation, USA) (30 m long × 0.25 mm internal diameter, 0.25 µm film thickness). Approximately 8.0 mg of dried supernatant sample were filled with 100 µl N,N-bis(trimethyl-silyl)trifluoro-acetamide in a clean, dry reaction vial. The mixture was vortexed for 5 min, incubated for 15 min at 70°C and filtered (0.22 µm polytetrafluoroethylene (PTFE) membrane filter) before automatic injection at 1 µl into the GC–MS machine using splitless mode. The inlet temperature was 200°C. The column oven temperature profile was pre-set as follows: 90°C at 0 min then ramped up to 280°C at a rate of 5°C min⁻¹ and held for 10 min. Helium was used as carrier gas at a flow rate of 1.5 ml min⁻¹.

**Gel permeation chromatography**

Polyhydroxyalkanoate sample was dissolved in tetrahydrofuran (THF) at a concentration of 2.0 mg ml⁻¹ and was filtered through 0.22 µm PTFE filter. Then, 100 µl of the sample was injected at 40°C with THF as mobile phase at a flow rate of 1.0 ml min⁻¹. The recorded chromatogram relative to the calibration curve of standard monodisperse polystyrene [(162, 380, 1,020, 1,320, 2,930, 6,770, 13,030, 29,150, 51,150, 113,300, 215,000, and 483,400 g mol⁻¹) (EasiVials, Agilent)] was obtained on Agilent LC 1220 (Agilent, USA) instrument equipped with a refractive index detector (Model 1260) and equipped with a refractive index detector (Model 1260). The machine was equipped with Mixed-D gel column (7.8 mm internal diameter × 300 mm) connected to MiniMix-D gel column (5 mm internal diameter × 25 mm). The gel permeation chromatography measurements were used to characterize the average weight molecular weight (Mw), number average molecular weight (Mn), and the polydispersity index of polymer.

**Thermogravimetric analysis**

Approximately 8–10 mg of the PHA sample was loaded onto the ceramic crucible pan. The sample was heated from 30 to 800°C at a rate of 10°C min⁻¹ under a nitrogen gas flow rate of 20 ml min⁻¹. The analysis was performed on a PerkinElmer thermogravimetric analysis 4000 instrument (PerkinElmer, USA).

**¹H NMR analysis**

¹H NMR analyses was performed by dissolving 5 mg PHA sample in 2 ml deuterated chloroform (CDCl₃) and filtered into NMR tube using borosilicate glass syringe equipped with 0.22 µm PTFE filter. This analysis was acquired using FT-NMR AVANCE III spectrometer (Bruker, New Zealand) at 400 MHz with tetramethylsilane as internal reference.

**Statistical analysis**

All measurements were conducted in triplicate. An analysis of variance was used to evaluate the significance of results, and p < 0.05 was considered to be statistically significant.

**Calculations**

i. \[
\text{%PHA content} = \frac{\text{PHA mass}}{\text{Total biomass (PHA mass + PHA - free cell mass)}} \times 100
\]

ii. \[
\text{%PHA depolymerized} = \frac{\text{PHA mass before depolymerization} - \text{PHA mass after depolymerization}}{\text{PHA mass in biomass before depolymerization}} \times 100
\]

iii. \[
\text{Average volumetric rate of depolymerization (g L⁻¹ h⁻¹)} = \frac{\Delta \text{PHA mass}}{\Delta \text{time}}
\]

iv. \[
\text{Percentage of liberated monomer} (\mu\text{mol L}⁻¹) = \frac{M_0 - M_t}{M_0} \times 100
\]

where \(M_0 = \text{total mass of monomer; } M_t = \text{monomer mass at time } t\).

v. \[
\text{%Yield} = \frac{\text{Actual yield}}{\text{Expected yield}} \times 100\%
\]

**Results and discussion**

**Preparation of crude reaction sample for in vitro depolymerization of intracellular mcl-PHA**

Figure 2 shows that liberation of cell protein content reached its maximum at 0.61 mg ml⁻¹ concentration following 120 min of ultrasonication. The cell shape was observed before (Figure 3a–c) and after lysis (Figure 3d–f) using confocal laser microscope under bright field and fluorescent imaging. Nile Red dye was used to stain intracellular mcl-PHA granules.
The fluorescent dye showed high affinity toward intracellular lipid-like material such as PHA thus enabling direct observation to be made under fluorescence microscopy. Both protein and microscopic analyses showed that the cells were completely lysed concomitantly releasing cellular content such protein after 120 min of ultrasonication. From micrographs taken before and after ultrasonication (Figure 3a–f), the general appearance of PHA granules released from lysed cells were observed to remain relatively intact.

**Effects of different sonication times on in vitro depolymerization of intracellular mcl-PHA**

The results obtained in Figure 4 showed different sonication times had significant effect toward mcl-PHA depolymerization activities. The highest amount of *in vitro*
Depolymerization of intracellular mcl-PHA, i.e., 51.25 ± 5.62 wt% [1.79 ± 0.05 g L⁻¹ from initial PHA content 3.51 ± 0.02 g L⁻¹ (Table 1)] was obtained from crude preparation of lysed cells following exposure to ultrasonication for 120 min. The lower mcl-PHA depolymerization in crude preparation obtained from cell suspension exposed to 90 min of sonication could be due to less efficient rupture of the cells. Exposure to ultrasound irradiation for 150 min could have denatured the depolymerization machinery of the mcl-PHA granules resulting in reduced depolymerization percentage (Figure 4). It is also likely that exposure to ultrasound irradiation for 150 min or more may decrease the susceptibility of the granules to enzymatic hydrolysis. Hence, 120 min was selected as the appropriate sonication time for the preparation of crude reaction sample exhibiting high mcl-PHA depolymerization activities. To eliminate the possibility that in vitro depolymerization could be due to direct effect of ultrasound, control whole cells with intracellular mcl-PHA and suspended in Tris-HCl buffer were exposed to thermal treatment at 70°C for 10 min prior to ultrasonication. The heat-treated cells were expected to exhibit insignificant in vitro depolymerization activities. Following ultrasonication at different times, the crude preparations of lysed cells were analyzed for depolymerization activities during 48-h incubation and they were found to be absent. The mcl-PHA content of the crude preparation remained constant at 37.4 ± 1.0 wt% throughout the experiment, and the percentage also represented an initial mcl-PHA content prior to ultrasound irradiation at different times. The absence of depolymerization activities was attributed to the thermal-related loss of enzymatic activities responsible for the in vitro action toward the mcl-PHA.

**Comparison of in vivo and in vitro depolymerizations of intracellular mcl-PHA**

A comparison between in vivo and in vitro depolymerizations of intracellular mcl-PHA was made. Bacterial cell culture after 48 h of cultivation in the production medium was collected by centrifugation and resuspended in buffer solutions of 0.2 M Tris-HCl, pH 9 and I = 0.2 M at 30°C for in vivo depolymerization of intracellular mcl-PHA (according to previous research). On the other hand, crude preparation from ultrasonication of cell suspension was collected and added to identical buffer (final volume 100 ml) and conditions for in vitro depolymerization of intracellular mcl-PHA. The depolymerization profiles were shown in Figure 5a and b and Tables 2 and 3 for in vivo and in vitro processes respectively. In vitro process showed lower rate and enzymatic depolymerization activities compared to the in vivo. The percentage of in vivo intracellular PHA depolymerization attained 92.2 ± 3.3 wt% whilst only 63.3 ± 1.0 wt% for in vitro process after 24 h incubation. The average initial content of mcl-PHA in both preparations ranged from 33.6 ± 2.0 wt% (1.9 ± 0.1 g L⁻¹) to 38.3 ± 2.0 wt% (2.3 ± 0.6 g L⁻¹). The total biomass weight declined with the decreasing mcl-PHA content in the cells after incubation for 48 h for in vivo process (5.94 ± 0.1–1.17 ± 0.0 g L⁻¹). For in vitro sample preparation, the initial cell concentration was measured at 7.65 g L⁻¹, and 5.73 ± 0.1 g L⁻¹ of cells were

**Table 1.** Effects of sonication time on in vitro depolymerization of intracellular mcl-PHA.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Initial PHA (g L⁻¹)</th>
<th>Final PHA (g L⁻¹)</th>
<th>Total PHA depolymerized (g L⁻¹)</th>
<th>% PHA depolymerized</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>3.32 ± 0.05</td>
<td>2.26 ± 0.01</td>
<td>1.06 ± 0.02</td>
<td>32.15 ± 2.90</td>
</tr>
<tr>
<td>120</td>
<td>3.51 ± 0.02</td>
<td>1.72 ± 0.05</td>
<td>1.79 ± 0.05</td>
<td>51.25 ± 5.62</td>
</tr>
<tr>
<td>150</td>
<td>3.45 ± 0.01</td>
<td>2.01 ± 0.05</td>
<td>1.44 ± 0.03</td>
<td>41.74 ± 0.40</td>
</tr>
</tbody>
</table>

Values are means of three replications ± standard deviation.

**Figure 4.** Effects of sonication time on in vitro depolymerization of intracellular mcl-PHA. Values are means of three replications ± standard deviation.

**Figure 5.** (a) Time profiles for in vivo depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and I = 0.2 M at 30°C. Values are means of three replications ± standard deviation. (b) Time profiles for in vitro depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and I = 0.2 M at 30°C. Values are means of three replications ± standard deviation.
enzymatic depolymerization activities of 0.88–4.61 U L\(^{-1}\) for C\(_{12}\), and at 24 h of incubation for C\(_{8}\) and C\(_{10}\), after which subunit composition was as follows: 40.06 ± 6.12 mol% of monomer distribution within the initial mcl-PHA with the highest rate of monomer liberation was observed for C\(_{6}\) followed by C\(_{10}\), C\(_{12}\), and C\(_{6}\). The initial polymer subunit composition was as follows: 40.06 ± 2.74 mol% C\(_{6}\); 32.78 ± 2.83 mol% C\(_{10}\); 21.04 ± 3.15 mol% C\(_{12}\), and 6.12 ± 2.04 mol% C\(_{6}\) subunits. The direct products of depolymerization were identical for both in vivo and in vitro processes. For in vivo process, the cells initiated immediate depolymerization of intracellular mcl-PHA to liberate the available monomers and reached plateau at 12 h for C\(_{6}\) and C\(_{12}\), and at 24 h of incubation for C\(_{8}\) and C\(_{10}\), after which no further changes were observed i.e., mcl-PHA hydrolysis had ceased. In contrast, for in vitro process, the crude preparation of mcl-PHA exhibited a more gradual depolymerization rate within 48 h of incubation to release all types of available monomers.

The comparison between average volumetric rates of in vivo and in vitro intracellular mcl-PHA depolymerizations and apparent depolymerization enzyme activities toward different monomers in 0.2 M Tris-HCl, pH 9 and \(I = 0.2\) M at 30°C was made in Table 4. The rate of in vivo mcl-PHA depolymerization was 0.15 ± 0.02 g L\(^{-1}\) h\(^{-1}\) equivalent to 11.98 ± 0.09 U L\(^{-1}\) apparent enzymatic depolymerization activity within 12 h of incubation. In contrast, the rate of in vitro mcl-PHA depolymerization was 0.06 ± 0.02 g L\(^{-1}\) h\(^{-1}\) equivalent to 4.79 ± 0.09 U L\(^{-1}\) apparent enzymatic depolymerization activity within 12 h of incubation. In addition, the rate of liberation of different monomers in vivo ranged from 0.01 to 0.06 g L\(^{-1}\) h\(^{-1}\) corresponding to apparent enzymatic depolymerization activities of 0.88–4.61 U L\(^{-1}\). Conversely, lower rate of liberation of different monomers for in vitro process was determined i.e., ranging from 0.00 to 0.02 g L\(^{-1}\) h\(^{-1}\) corresponding to apparent enzymatic depolymerization activities of 0.46–1.94 U L\(^{-1}\).

In other reported studies, in vitro depolymerization of intracellular nPHA had been investigated using granules isolated by glycerol density gradient centrifugation as well as

### Table 2. Time profiles for in vivo depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and \(I = 0.2\) M at 30°C.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Dry cell weight (g L(^{-1}))</th>
<th>PHA content (%)</th>
<th>PHA content (g L(^{-1}))</th>
<th>% PHA de-polymerized</th>
<th>Total PHA de-polymerized (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.94 ± 0.07</td>
<td>38.27 ± 2.02</td>
<td>2.27 ± 0.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>2.38 ± 0.04</td>
<td>19.64 ± 1.06</td>
<td>0.47 ± 0.05</td>
<td>79.43 ± 2.86</td>
<td>1.81 ± 0.02</td>
</tr>
<tr>
<td>24</td>
<td>1.63 ± 0.20</td>
<td>10.93 ± 1.22</td>
<td>0.18 ± 0.02</td>
<td>92.15 ± 3.30</td>
<td>2.10 ± 0.01</td>
</tr>
<tr>
<td>36</td>
<td>1.28 ± 0.07</td>
<td>4.39 ± 0.51</td>
<td>0.06 ± 0.02</td>
<td>97.51 ± 1.36</td>
<td>2.22 ± 0.01</td>
</tr>
<tr>
<td>48</td>
<td>1.17 ± 0.02</td>
<td>2.75 ± 0.25</td>
<td>0.03 ± 0.01</td>
<td>98.57 ± 1.32</td>
<td>2.24 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means of three replications ± standard deviation.

### Table 3. Time profiles for in vitro depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and \(I = 0.2\) M at 30°C.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total pelleted fraction weight (g L(^{-1}))</th>
<th>PHA content (%)</th>
<th>PHA content (g L(^{-1}))</th>
<th>% PHA de-polymerized</th>
<th>Total PHA de-polymerized (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.73 ± 0.22</td>
<td>33.60 ± 2.00</td>
<td>1.93 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>4.42 ± 0.32</td>
<td>28.56 ± 2.20</td>
<td>1.27 ± 0.08</td>
<td>34.29 ± 4.50</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>24</td>
<td>2.61 ± 0.30</td>
<td>26.95 ± 1.00</td>
<td>0.71 ± 0.10</td>
<td>63.33 ± 1.02</td>
<td>1.22 ± 0.08</td>
</tr>
<tr>
<td>36</td>
<td>2.09 ± 0.08</td>
<td>21.17 ± 1.50</td>
<td>0.44 ± 0.10</td>
<td>77.04 ± 1.58</td>
<td>1.48 ± 0.01</td>
</tr>
<tr>
<td>48</td>
<td>1.75 ± 0.05</td>
<td>16.50 ± 0.45</td>
<td>0.29 ± 0.01</td>
<td>85.03 ± 4.60</td>
<td>1.64 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means of three replications ± standard deviation.

![Figure 6](image-url)
centrifuged crude cell extracts without glycerol and incubating the obtained polymer fraction with hydrolytic enzymes methods, or making use of artificial PHB granules prepared according to the procedure described previously by Horowitz and Sanders. Several factors have been studied to improve the in vitro depolymerization rate of nPHA e.g., by adding Mg2+ ions to increase ionic strength, pretreatment nPHA granule or trypsin or protease prior to subsequent hydrolysis by soluble iPHA depolymerase, or adding freshly soluble cell extract to nPHA. In contrast, the current study utilized cell-free biology of the crude preparation of ultrasonicated-lysed cells containing nPHA, iPHA depolymerase and other hydrolase enzymes, and more importantly the depolymerization of mcl-PHA to generate R3HAs was highly active in the in vitro system despite lower depolymerization rate compared to the in vivo whole cells process. The real advantage of the presented in vitro system as a potential route for chiral R3HAs production is the absence of multi-step pre-treatments on the crude reaction mixture obtained from mcl-PHA accumulating bacterial cell culture.

**Characterization**

**Gel permeation chromatography**

Molecular weight data for in vivo and in vitro depolymerizations of intracellular mcl-PHA are shown in Table 5. Both processes exhibited decreasing molecular weight after depolymerization. The results showed that the average molecular weights \(M_n\) and \(M_w\) after in vitro process were lower than after in vivo. In addition, the polydispersity \(M_w/M_n\) index increased after in vitro depolymerization compared to initial samples and after in vivo depolymerization. The modest decrease in the molecular weight and the constant polydispersity of intracellular mcl-PHA during in vivo process could indicate an exo-type reaction which hydrolyzes ester bond of constituent monomers starting from terminal-end of polymer chain on the granule surface resulting in high molecular weight fragments inside the granule subsequent to the initiation of depolymerization. Conversely, for in vitro process the marked decrease in the average molecular weights and the higher polydispersity indicated that the liberated mcl-PHA samples from lysed cells were present as fragments with relatively wide molecular weight distribution. The possibility that ultrasound exposure may contribute to the fragmentation is not being ruled out at this stage. Nevertheless, depolymerization enzyme activities could still attack the fragments from their terminal end thus explaining the presence of single monomer types as the only detectable exogenous direct products from mcl-PHA depolymerization in the cell debris-free supernatant of in vitro process (further discussion in the next section).

**Thermogravimetric analysis**

Decomposition temperature for dried sample preparations containing mcl-PHA after in vivo and in vitro processes was determined from thermal analysis without the samples undergoing chloroform extraction. Control samples (thermally-treated whole cells with intracellular mcl-PHA; 70°C for 10 min) and untreated cell samples without intracellular mcl-PHA were used as references. As can be seen in Figure 7, both samples from in vivo and in vitro processes showed weight lost in the temperature range of 250–500°C at decomposition temperatures \(T_d\) of 264.9 and 266.0°C respectively. However, the \(T_d\) for untreated cell without mcl-PHA was lower at 246.3°C whereas \(T_d\) for control samples was higher at 287.9°C. It showed that the samples after in vivo and in vitro depolymerizations contained less polymer compared to control samples (thermally treated whole cells with intracellular mcl-PHA) following depolymerization of intracellular mcl-PHA.

**\(^1\)H NMR**

The spectra (Figure 8) showed signals corresponding to the protons of the extracted mcl-PHA samples before depolymerization, and after in vivo and in vitro processes in Tris-HCl, pH 9 and \(I = 0.2\) M at 30°C. The \(^1\)H NMR spectra after both processes were very similar to the neat mcl-PHA with signal

| Table 4. Average volumetric rate of in vivo and in vitro depolymerizations of intracellular mcl-PHA and apparent enzymatic depolymerization activities toward different monomers in 0.2 M Tris-HCl buffer, pH 9, during 12 h incubation. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                 | Mcl-PHA         | C₆-Hx          | C₇-HO          | C₁₀-HD         | C₁₂-HDD        |
| **In vivo intracellular depolymerization** |                   |                 |                |                |                |
| Initial (g L⁻¹)                | 2.27 ± 0.20     | 0.10 ± 0       | 0.93 ± 0.03    | 0.82 ± 0.02    | 0.41 ± 0.02    |
| Final (g L⁻¹)                  | 0.47 ± 0.11     | 0.02 ± 0       | 0.20 ± 0.02    | 0.16 ± 0.03    | 0.09 ± 0.01    |
| Average volumetric rate of depolymerization (g L⁻¹ h⁻¹) | 0.15 ± 0.02     | 0.01 ± 0.00    | 0.06 ± 0.01    | 0.06 ± 0.01    | 0.03 ± 0.00    |
| Apparent depolymerization activity (U L⁻¹) | 11.98 ± 0.09    | 0.88 ± 0.01    | 4.61 ± 0.05    | 4.52 ± 0.02    | 1.97 ± 0.01    |
| **In vitro intracellular depolymerization** |                   |                 |                |                |                |
| Initial (g L⁻¹)                | 1.93 ± 2.4      | 0.09 ± 0       | 0.71 ± 0.01    | 0.65 ± 0.01    | 0.48 ± 0       |
| Final (g L⁻¹)                  | 1.27 ± 4.7      | 0.05 ± 0       | 0.47 ± 0       | 0.44 ± 0       | 0.31 ± 0       |
| Average volumetric rate of depolymerization (g L⁻¹ h⁻¹) | 0.06 ± 0.02     | 0.00 ± 0       | 0.02 ± 0       | 0.02 ± 0       | 0.01 ± 0       |
| Apparent depolymerization activity (U L⁻¹) | 4.79 ± 0.09     | 0.46 ± 0.01    | 1.94 ± 0.05    | 1.43 ± 0.02    | 0.97 ± 0.01    |

Values are means of three replications ± standard deviation.
peaks from both spectra displaying almost identical chemical
shifts to the spectra obtained from previous research on
bacterial mcl-PHA production using lauric acid. From the
representative spectrum, the multiplet peaks at 2.5 ppm and
triplet peaks at 5.2 ppm were assigned to methylene and meth-
ine protons of the α- and β-carbon, respectively. The chemical
shifts at 0.9 and 1.2 ppm were assigned to the terminal methyl
and methylene protons in the side chain of the polymers
respectively. Chemical shift at 1.6 ppm was attributed to
methylene protons adjacent to the β-carbon in the side chains
of 3-hydroxyalkanoates (3HA) copolymer.[9,25,29,34] The signal
intensity ratio of proton methine (5.2 ppm) group to proton
methyl (0.9 ppm) group was reduced after in vivo and in vitro
derpolymerizations, which supported the occurrence of ester
linkages hydrolysis in mcl-PHA by enzymatic depolymeriza-
tion activities.[9,34]

**Profiling of exogenous direct products from in vivo and
in vitro depolymerizations of mcl-PHA**

The water-soluble compounds of exogenous direct products
from both in vivo and in vitro depolymerizations were
subjected to silylation for GC–MS analysis. Silylation is the
most established derivatization method as it readily volatizes
the non-volatile sample by the addition of silyl groups to the
free –OH groups. The silylated derivatives are more volatile
and more stable for GC analysis.[35] Four major peaks
corresponding to main products of in vivo and in vitro
processes were successively obtained from GC–MS analysis
as 3-trimethylsiloxy esters of; hexanoic (m/z: 276), octanoic
(m/z: 304), decanoic (m/z: 332) and dodecanoic (m/z: 360)
acids. The ion impact mass spectra of 3-trimethylsiloxy
esters are shown in Table 6. All products were detected in
the form of single type monomers only. The detection of
single 3HA monomers that matched the composition of
nPHA in the cell-free supernatant has important implication
that it supported the hypothesis of exo-type reaction for
intracellular mcl-PHA hydrolysis by enzymatic depolymeri-
zation activities. Chen et al.[17] also reported that almost all
the in vitro hydrolytic products obtained from the depoly-
merization of intracellular amorphous PHA by Bacillus
megaterium are 3HB monomers. The yield of each of the
four 3HA monomers was also calculated based on the spec-
tral data obtained from the GC–MS analysis (Table 7). Inter-
estingly, while the rate of depolymerization was consistently
higher for all types of monomers in in vivo process (Table 4),
significantly higher yield of monomer depolymerization
was observed for in vitro process (Table 7), with complete
or near complete liberation of 3HD and 3HHx/3HO
monomers, respectively. It is likely that in in vivo process,
the competing metabolic pathway(s) may play important
role in diverting away the yield since living whole cells were
used. It also showed that whilst the in vitro process exhibited
lower rate of depolymerization than the in vivo, the yields
were comparatively better thus more attractive to be applied.
for the production of various types of mcl (R)-3HAs in a cell-free biology system.

**Conclusion**

The study showed that both *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA could be a potential biological route for the production of various types of mcl (R)-3HAs as platform chemicals. Its primary advantage is the absence of complicated and often non environmental-friendly multistep upstream processes.

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**References**


