Flux balance analysis for maximizing polyhydroxyalkanoate production in *Pseudomonas putida*

S Ramalingam*, M Vikram, M P Vigneshbabu and M Sivasankari
Center for Biotechnology, Anna University, Chennai 600 025, India

The aim of this work is to develop process strategies to understand the behaviour of *Pseudomonas putida* for cost-effective synthesis of mcl-PHA with the help of metabolic flux model developed using linear programming. After screening and selection from various carbon sources in shake flask experiments, 2 carbon sources, namely, glucose and glycerol were selected for further studies. The possibilities for higher PHA biosynthesis were illustrated by the flux balance analysis (FBA) supplemented with continuous cultivation data. The optimal ratio between glucose, glycerol and nitrogen is obtained by simulation of the metabolic model. But the cost of raw materials is the tailback for the cost-effective production of mcl-PHA. Hence, a metabolic flux distribution model was developed for dual substrate utilization by co-feeding glucose and glycerol for optimal PHA synthesis. Response surface results indicated that the highest PHA accumulation conditions for glucose and glycerol.

**Keywords**: Biopolymers, chemostat, C/N ratio, flux balance analysis, PHA, *Pseudomonas putida*

**Introduction**

Polyhydroxyalkanoates (PHA) are biopolymers stored by a wide variety of organisms as an energy reserve. There were two major factors that eventually brought upon the PHA a widespread industrial interest: 1) The world oil crisis in the early 1970s that resulted in the instability of oil market and oil shortages; and 2) A growing interest in biodegradability and sustainability of materials in the 1980s and early 1990s. PHA being biodegradable and biocompatible has application that varies from industries to medical therapeutics. Microorganisms accumulate PHA as an intracellular reserved material in response to the imbalance in the growth environment where a suitable carbon source is present in excess and one or more nutrients are limiting, e.g., nitrogen, oxygen, phosphorus, iron, magnesium, manganese, potassium and sodium, or when the C:N ratio of the feed substrate is high, an excess of carbon source with deficiency in trace elements, etc. Metabolic flux analysis is the method to evaluate the amount of intracellular metabolites flow in the central carbon metabolism by the knowledge of rate of production of extracellular products, rate of substrate utilization, rate of product formation, rate of biomass production, etc. Simulations performed in this work throw light on the flux distribution during optimum conditions and also on the cost-effective combination of carbon sources.

**Materials and Methods**

All chemicals, reagents and medium components used were of analytical grade. Standard reagents and medium components including glucose and inorganic salts were obtained either from Merck & Co. (New Delhi, India) or Himedia Laboratories (Mumbai, India).

**Strain and Media Used**

*Pseudomonas putida* MTCC 102 (type B), a Gram-negative bacterium obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India, was used throughout the study. Two types of media—i) a rich medium (Hori et al 1994) primarily for promoting biomass growth, and ii) A defined medium has been used as the basal medium for PHA production—were used to culture the bacterium.
High Performance Liquid Chromatography (HPLC)

The carbon sources and various extracellular metabolites were quantitatively estimated using HPLC equipped with Aminex HPX-87H column and Refractive Index Detector. Prior to metabolites estimation in the fermenter broth, the standard graphs of metabolites like citric acid, succinic acid, lactic acid and acetic acid were prepared from different concentrations of each metabolite and their corresponding area under the peak. The amount of carbon sources and metabolites like acetic acid, lactic acid, citric acid, succinic acid and ethanol were analyzed quantitatively during fermentation.

Fourier-transform Infrared Spectroscopy (FTIR)

FT-IR has been demonstrated to be a powerful tool for studying microorganisms and their cell components in intact form. In this study, the extended observation was done to detect mcl-PHA by the FT-IR technique in intact cells and also in purified form.

Purification and Measurement of PHA from Biomass

The purification procedure was used for PHA extraction was described earlier. Total PHA content is the ratio of the total amount of polymer obtained after final purification by methanol precipitation to the weight of the dried cells. It was found that the percentage of recovery was not affected by the presence of residual biomass and the same has been mentioned.

Flux Balance Model

The full genome sequence of P. putida is available with functional annotation in online databases like NCBI. The pathways active in the organism were listed from KEGG database. Some missing enzymes were cross checked by blasting the protein sequence against the genome. Finally, the published literature data of the full genome model was used for confirmation. The model included 134 reactions and 121 metabolites. Hence, it gave 121 flux equations and 134 flux variables with 13 degrees of freedom. The system was then formulated in a linear programming problem with the objective function of maximizing biomass. The modeling was done on the lines of the flux balance methodology described by Edwards et al.

Optimization Using Linear Programming by GAMS

The flux analysis was performed with the help of the General Algebraic Modeling System (GAMS), a modeling system for mathematical programming and optimization of objective of interest, particularly designed for modeling linear, non-linear and mixed integer optimization problems, wherein linear programming (LP) is a technique for optimization of a linear objective function to achieve the best outcome in a given mathematical model (here GAMS) subject to linear equality and linear inequality constraints.

Results

Confirmation of mcl-PHA Biosynthesis by FTIR Analysis

FTIR analysis is one of the accurate methods for the detection of PHA in intact cells or in purified PHA. The FT-IR method does not require extensive sample preparation and, therefore, it is also very useful for broad screening of PHA producing microorganisms. Hong et al. developed a rapid method for detecting bacterial polyhydroxyalkanoates both in intact cells and purified form by Fourier-transform infrared spectroscopy.

The FTIR was done and the bands observed in the FTIR spectra (Fig. 1) were found similar to the bands observed in the spectra reported by Hong et al. In total, 4 bands corresponding to mcl-PHA were observed and they were at wave numbers’ 1740.44, 2859.92, 1069.33 and 2924.52. The obtained FT-IR spectrum confirms the presence of mcl-PHA (Fig. 1). The four peaks of the FTIR spectra shown were similar to the peaks obtained by Hong et al. The first three bands confirm the presence of mcl-PHA and the last band describes the methylene C-H vibration of mcl-PHA as it is also observed by Hong et al.

PHA from Different Carbon Sources

Screening for higher PHA biosynthesis using various carbon sources was carried out. P. putida was cultured in basal medium supplemented with one of...
the following as the sole carbon source (10 g/L). This includes carboxylic acids (fatty acids), carbohydrates, straight chain alkanes (n-pentane and n-octane) and glycerol. The cells were cultivated in shake flasks. The stationary phase culture was analyzed for the amount of biomass produced and PHA synthesized (Table 1). A difference of 7-fold was observed between the various carbon sources. Amongst the carbohydrates, glucose produced 9% PHA from its total cell dry wt, straight chain alkanes produced relatively less amounts of PHA and glycerol produced 15% PHA. Therefore, glucose and glycerol were selected for further *in silico* study.

### Continuous Cultivation Studies

The growth profile of the culture during transient state was followed by steady state. Nearly four reactor volume (5.6 L) of the medium was fed to achieve steady state. Steady state in the reactor was identified by the steady state cell concentration, residual substrates and various other secreted metabolites in the culture broth. The steady state was maintained for 20 h to have reliable output. The carbon source concentration of 10 g/L was chosen as it was not inhibitory to growth in the shake flask and batch experiments. In order to analyze the metabolic flux for these different carbon sources at the same dilution rate and to prevent the washout, a dilution rate of 0.06/h was arbitrarily chosen for the experiments.

Analyses of the various extracellular metabolites, which are the by-products at steady state, are shown in Table 2. The metabolites detected by HPLC for glucose as carbon source were citric acid, lactic acid, acetic acid, succinic acid and ethanol; whereas on glycerol, succinic acid and ethanol are the two metabolites secreted. Continuous fermentation experiments with various limiting nutrients like N, P, Mg, and Fe has been done and found that PHA production increased significantly, when nitrogen was used as a limiting nutrient. The nitrogen limitation resulted in redirecting the carbon flux towards mcl-PHA biosynthesis. Therefore, nitrogen was used as the limiting source in chemostat experiments.

### Validation of Model

The experimental verification of the predictions of the model was done for the various sets of the input data. A comparison was done between the predicted and experimental values as shown in the Table 3. The modifications necessary for fitting the values obtained in different experimental trials were incorporated. Now the model was validated for their predictive capability which was well above 90% in all cases.

### Simulation of Dual Substrate Metabolic Flux Model

A metabolic flux model with simultaneous utilization of glucose and glycerol was developed. The ratio of glucose and glycerol was varied as shown in the Table 4. This table of simulated values was then

---

**Table 1**—Shake flask experiments done for 19 carbon substrates with triplicates

<table>
<thead>
<tr>
<th>Carbon substrate</th>
<th>CDW g/L</th>
<th>PHA content g/L</th>
<th>PHA yield %</th>
<th>Polymer nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>3.23 ± 0.05</td>
<td>1.14 ± 0.05</td>
<td>35.6</td>
<td>Sticky</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2.66 ± 0.05</td>
<td>0.82 ± 0.02</td>
<td>31</td>
<td>Sticky</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>0.68 ± 0.03</td>
<td>0.22 ± 0.03</td>
<td>33.1</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>Tween-20 (mono laurate)</td>
<td>0.49 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>26.3</td>
<td>Sticky</td>
</tr>
<tr>
<td>Tween-80 (mono oleate)</td>
<td>0.44 ± 0.05</td>
<td>0.08 ± 0.01</td>
<td>19.1</td>
<td>Sticky</td>
</tr>
<tr>
<td>Saponified sesame oil</td>
<td>3.35 ± 0.03</td>
<td>0.62 ± 0.05</td>
<td>18.5</td>
<td>Sticky</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>0.52 ± 0.05</td>
<td>Trace amount</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sesame oil</td>
<td>0.95 ± 0.02</td>
<td>0.14 ± 0.03</td>
<td>15</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>2.87 ± 0.07</td>
<td>0.80 ± 0.02</td>
<td>28</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>n-Octanoic acid</td>
<td>1.25 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>5.9</td>
<td>Sticky</td>
</tr>
<tr>
<td>n-Pentane</td>
<td>0.43 ± 0.05</td>
<td>Trace amount</td>
<td>-</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>n-Octane</td>
<td>1.0 ± 0.04</td>
<td>0.1 ± 0.02</td>
<td>9.8</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.99 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>9.0</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.90 ± 0.04</td>
<td>Trace amount</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>1.82 ± 0.04</td>
<td>0.09 ± 0.01</td>
<td>5.3</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.71 ± 0.03</td>
<td>0.18 ± 0.01</td>
<td>6.7</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.24 ± 0.05</td>
<td>0.09 ± 0.02</td>
<td>7.8</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Malate</td>
<td>0.96 ± 0.03</td>
<td>Trace amount</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.81 ± 0.04</td>
<td>0.57 ± 0.03</td>
<td>15.1</td>
<td>Amorphous</td>
</tr>
</tbody>
</table>

**Table 2**—Rate of formation of extracellular metabolites and mcl-PHA for various carbon sources in nitrogen limiting continuous fermentation

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Acetic acid (mmole/g/h)</th>
<th>Citric acid (mmole/g/h)</th>
<th>Ethanol (mmole/g/h)</th>
<th>Lactic acid (mmole/g/h)</th>
<th>Succinic acid (mmole/g/h)</th>
<th>mcl-PHA (mmole/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.2 × 10⁻³</td>
<td>1.95 × 10⁻³</td>
<td>0.0489</td>
<td>3.714 × 10⁻³</td>
<td>6.3 × 10⁻⁴</td>
<td>8.32 × 10⁻⁵</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>-</td>
<td>0.047</td>
<td>-</td>
<td>4.44 × 10⁻⁵</td>
<td>1.22 × 10⁻⁵</td>
</tr>
</tbody>
</table>
fitted into the quadratic equation using MATLAB. The quadratic equation obtained was:

\[ PHA = 0.817 + 0.003*\text{[Glu]} + 0.002*\text{[Gly]} - 0.024*\text{[N]} - 1.483e-5*\text{[Glu]}*\text{[Gly]} + 8.449e-05*\text{[Glu]}*\text{[N]} + 7.463e-05*\text{[Gly]}*\text{[N]} - 1.398e-05*\text{[Glu]}^2 - 1.082e-05*\text{[Gly]}^2 + 8.715e-05*\text{[N]}^2 \]

The equation was then plotted across the three dimensions, namely, the concentration of glucose, glycerol and nitrogen source as shown in Fig. 2.

**Discussion**

In chemostat cultivations, growth on glucose was found to be much faster than on glycerol. These observations suggest that glucose is the preferred carbon source for increasing the cell density. Production of PHA together with optimum amount of biomass is critical for successful large scale production. The flux model development resulted in minimizing the number of experiments needed to achieve the objective. It also helped in easy prediction of the optimum composition of the carbon and nitrogen sources required in the media for maximum product accumulation and growth.

The simulated space provided dark regions of good PHA accumulation, which increased with the decrease in nitrogen limitation. The highest possible N concentration could be chosen from within this high productivity region. Hence, *in silico* analysis, this gives an insight for the cost-effective production of mcl-PHA. Flux distribution maps provide idea about the possible gene manipulation targets. The effect of the knocked out or inserted gene on the amount of product formed can also be calculated.

In conclusion, multiple rounds of metabolic engineering might be essential to achieve higher product levels in glucose and glycerol. This work eliminates most of the initial steps required to be performed. Further work could be directed towards the elimination of the bottlenecks in the flux map, which can be easily elucidated using the flux balance model. Further, this methodology could be implemented for medium design and gene manipulation.

**References**

2. Suriyamongkol P, Weselake R, Narine S, Moloney M & Shah S, Biotechnological approaches for the production of


