การวิเคราะห์เมตาบอลิกฟลักซ์ของวิถีการสังเคราะห์โพลี (3-ไฮดรอกซีบิวทิเรต-*โค*-3-ไฮดรอกซีวาเลอเรต) ใน *Ralstonia eutropha* NCIMB 11599

นาย ณัฐวุฒิ ลักษณาปัญญากุล

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METABOLIC FLUX ANALYSIS OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) BIOSYNTHETIC PATHWAYS IN *RALSTONIA EUTROPHA* NCIMB 11599

Mr. Nuttawut Laksanapanyakul

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ณัฐวุฒิ ลักษณาปัญญากุล: การวิเคราะห์เมตาบอลิกฟลักซ์ของวิถีการสังเคราะห์โพลี(3-ไฮดรอกซีบิวทิเรต-โค-3-ไฮดรอกซีวาเลอเรต) ใน *Ralstonia eutropha* NCIMB 11599 (METABOLIC FLUX ANALYSIS OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) BIOSYNTHETIC PATHWAYS IN *RALSTONIA EUTROPHA* NCIMB 11599)
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งานวิจัยนี้มีจุดมุ่งหมายในการศึกษาการนำการวิเคราะห์เมตาบอลิกฟลักซ์ไปใช้สำหรับการสังเคราะห์ โพลี(3-ไฮดรอกซีบิวทิเรต-โค-3-ไฮดรอกซีวา-เลอเรต) ในแบคทีเรีย *Ralstonia eutropha* NCIMB 11599 โดยมีวัตถุประสงค์หลักคือ เพื่อใช้ในการพัฒนากระบวนการผลิตพลาสติกที่สามารถย่อยสลายได้ตามธรรมชาติ ให้ได้ผลิตภัณฑ์ที่เป็นไปตามความต้องการของตลาด กล่าวคือพลาสติกที่ผลิตได้จะต้องมีสัดส่วนของ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ อยู่ในปริมาณมากที่สุด โดยงานวิจัยนี้สามารถแบ่งออกได้เป็น 4 ขั้นตอนหลักๆ

ขั้นตอนแรก มีวัตถุประสงค์เพื่อเสนอแผนภาพจำลองวิถีการสังเคราะห์พลาสติกชนิดนี้ภายใต้สภาวะที่แตกต่างกัน คือ ชนิดของแหล่งคาร์บอนและระดับการจำกัดของไนโตรเจน จากการศึกษาพบว่า สามารถสร้างแผนภาพจำลองวิถีการสังเคราะห์พลาสติกได้ 4 แผนภาพ โดยทั้ง 4 แผนภาพมีความเหมือนกันเป็นส่วนใหญ่ ได้แก่ วิถีเพนโทสฟอสเฟต วิถีการสังเคราะห์อะซีติลโคเอนไซม์เอ วัฏจักรเครปส์ วิถีการสังเคราะห์ 3-ไฮดรอกซีบิวทิเรตโมโนเมอร์ วิถีการนำเข้าแอมโมเนียมและการผลิตเรซิดวลไบโอแมส วิถีการผลิตพลังงานออกซิเดทีฟฟอสโฟริเลชั่นและการหายใจ และวิถีอะแน็พเพลโรติกโดยเอนไซม์ฟอสโฟอีนอลไพรูเวต คาร์บอกซี่คิเนส ส่วนที่แตกต่างกัน ได้แก่ วิถีการย่อยสลายกลูโคส วิถีการย่อยสลายกรดโพรพิโอนิก วิถีการสังเคราะห์ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ และวิถีการสังเคราะห์ไพรูเวตจากมาเลตโดยเอนไซม์มาลิก

ขั้นตอนที่สอง มีวัตถุประสงค์เพื่อคำนวณหาค่าผลได้ของ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ทางทฤษฎีที่สูงที่สุด จากการศึกษาพบว่าค่าสัดส่วน 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ในโคโพลิเมอร์ทางทฤษฎีมีค่าแตกต่างจากค่าที่ได้จากการทดลองจริงอยู่มาก โดยค่าสูงสุดที่คำนวณได้ทางทฤษฎี มีค่า 75 เปอร์เซ็นต์ แต่ค่าสูงสุดที่ได้จากการทดลอง มีค่า 56 เปอร์เซ็นต์ ดังนั้น ค่าผลได้ของโมโนเมอร์ยังสามารถได้รับการปรับปรุงได้อีกมาก แต่การปรับปรุงดังกล่าวอาจจะต้องใช้การประยุกต์หลักการทางเมตาบอลิกเอ็นจิเนียริ่งมาใช้เป็นหลัก

ขั้นตอนที่สาม มีวัตถุประสงค์เพื่อ ชี้บ่งว่าวิถีสังเคราะห์ใดน่าจะเป็นขั้นตอนที่ขัดขวางการสังเคราะห์ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ ทำให้ประสิทธิภาพในการสังเคราะห์ต่ำกว่าค่าผลได้ของโมโนเมอร์ทางทฤษฎีที่สูงที่สุดที่คำนวณได้จากขั้นตอนที่สอง จากการศึกษาพบว่า โนดที่สำคัญที่มีผลต่อวิถีการสังเคราะห์พลาสติกนี้มี 2 โนด คือ ที่โนดอะซีติลโคเอนไซม์เอ และโนดโพรพิโอนิลโคเอนไซม์เอ ดังนั้นวิถีการสังเคราะห์รอบๆ โนดนี้น่าจะเป็นขั้นตอนคอขวดที่ขัดขวางการสังเคราะห์ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ หลักในการแก้ไขขั้นตอนคอขวดนั้นได้แก่ 1) เพิ่มฟลักซ์ของอะซีติลโคเอนไซม์เอที่จะมารวมตัวกับโพรพิโอนิลโคเอนไซม์เอ เพื่อเป็นการเพิ่มการสังเคราะห์ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ 2) ลดฟลักซ์ของอะซีติลโคเอนไซม์เอที่จะรวมตัวกันเอง เพื่อเป็นการลดการสังเคราะห์ 3-ไฮดรอกซีบิวทิเรตโมโนเมอร์ 3) เพิ่มฟลักซ์ของโพรพิโอนิลโคเอนไซม์เอที่จะมารวมตัวกับอะซีติลโคเอนไซม์เอ เพื่อเป็นการเพิ่มการสังเคราะห์ 3-ไฮดรอกซีวาเลอเรตโมโนเมอร์ และ 4) ลดฟลักซ์ของโพรพิโอนิลโคเอนไซม์เอที่แยกเข้าสู่วัฏจักรกรดเมทิลซิตริก

และขั้นตอนสุดท้าย เป็นการเสนอแนะวิธีในการแก้ไขวิถีสังเคราะห์ที่มีปัญหานั้นๆ ตามหลักการเมตาบอลิกเอ็นจิเนียริ่ง โดยงานวิจัยนี้มีข้อเสนอแนะดังต่อไปนี้ 1) เพิ่มแอคทีฟไซต์ของเอนไซม์ 3-คีโตไทโอเลส ให้มีความเฉพาะเจาะจงกับโพรพิโอนิลโคเอนไซม์เอมากกว่ากับอะซีติลโคเอนไซม์เอ 2) เพิ่มค่ากิจกรรมของเอนไซม์พีเอชบีซินเทส 3) ลดหรือลบเอนไซม์ในวัฏจักรเครปส์ 4) ลดหรือลบเอนไซม์ในวัฏจักรกรดเมทิลซิตริก 5) เพิ่มค่ากิจกรรมของเอนไซม์กลูโคส-6-ฟอสเฟตดีไฮโดรจีเนส เพื่อเพิ่มปริมาณนิโคตินาไมด์อาเดนีนไดนิวคลีโอไทด์ฟอสเฟต และ 6) เพิ่มปริมาณโพรพิโอนิลโคเอนไซม์เอภายในเซลล์ให้สูงขึ้น โดยการเติมสารที่สามารถผลิตโพรพิโอนิลโค-เอนไซม์เอ หรือการเพิ่มวิถีการสังเคราะห์ที่นำไปสู่การสังเคราะห์โพรพิโอนิลโคเอนไซม์เอเพิ่มเติม

ภาควิชา วิศวกรรมเคมี
สาขาวิชาวิศวกรรมเคมี
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ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NUTTAWUT LAKSANAPANYAKUL: METABOLIC FLUX ANALYSIS OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) BIOSYNTHETIC PATHWAYS IN *RALSTONIA EUTROPHA* NCIMB 11599. THESIS ADVISOR: ASSISTANT PROFESSOR SEEROONG PRICHANONT, Ph.D. THESIS COADVISOR: ATSAWIN MEECHAI, Ph.D. , PP. ISBN 974-17-9758-3

This research is the study of application of metabolic flux analysis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3-HV)] biosynthesis in *Ralstonia eutropha* NCIMB 11599. The ultimate aim of this research is to improve the production process of P(3HB-co-3HV), a biodegradable plastic, to meet the commercial requirement that the copolymer contains the fraction of 3-hydroxyvalerate (3HV) monomeric unit as much as possible. To achieve so, the research is categorized into four main sections.

As for the first section, the purpose is to propose metabolic network that can describe the metabolic behavior of *R. eutropha* NCIMB 11599 when it is growing under various conditions, i.e., under different carbon sources and levels of nitrogen limitation. Accordingly, there are four metabolic networks. These pathways are depending upon used substrate and level of nitrogen limitation. Mainly, there are such similarities among them; namely, modified pattern of pentose-phosphate (PP) pathway, acetyl coenzyme A formation, tricarboxylic acid (TCA) cycle, 3-hydroxybutyrate formation route, ammonium assimilation via GS-GOGAT system, residual biomass formation, oxidative phosphorylation and respiratory pathway, and phosphoenolpyruvate (PEP) carboxykinase as the only route of anaplerotic pathway. As for the differences, there are at the catabolism of glucose and propionic acid, 3-hydroxyvalerate formation route, and NADPH-linked malic enzyme as an additional source of NADPH production.

In the second section, calculation of maximum theoretical 3HV molar fraction in the copolymer is carried out. It can be concluded that the maximum theoretical values (maximum at 75%) are higher than the ones computed from the experimental data (maximum at 56%). This suggests that there is still more room for improvement of 3HV fraction in the copolymer. Interestingly, the improvement can be systematically attained by the application of metabolic engineering.

Regarding the third section, identification of principle nodes and possible bottlenecks is accomplished. According to the analysis of flux split ratio, there are two principle nodes in the metabolic network where a mixture of glucose and propionic acid is used as a carbon source. These nodes are acetyl coenzyme A (AcCoA) and propionyl coenzyme A (ProCoA). Providing that the increased molar fraction of 3HV is desired, flux split ratios at these two nodes need to be modified since the fluxes around these two nodes are possible bottlenecks. The modifications can be achieved by; a) increasing the flux of AcCoA that condenses with ProCoA to form more 3HV; b) decreasing the flux of AcCoA that condenses to form less 3HB; c) increasing the flux of ProCoA that condenses with AcCoA to form more 3HV; and d) decreasing the flux of ProCoA that enters MCA cycle.

From the above conclusions, the target sites for metabolic engineering are suggested in the last section. as follows; a) engineering the active site of 3-ketothiolase for much more active with ProCoA comparing to AcCoA; b) amplifying PHB synthase; c) fully or partially blocking isocitrate dehydrogenase enzyme; d) fully or partially blocking methylcitrate lyase; e) amplifying glucose-6-phosphate dehydrogenase enzyme to produce more NADPH; and f) increasing intracellular ProCoA by feeding additional ProCoA-yielding substrate and/or introducing an additional ProCoA formation route.

Department Chemical Engineering Student's signature……..

Field of study Chemical Engineering Advisor's signature……..

Academic year 2002 Co-advisor's signature…….

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NOMENCLATURE

Symbol	Definition	Unit
$\overline{\overline{A}}$	Matrix containing the stoichiometric coefficients for the substrates	
$\overline{\overline{B}}$	Matrix containing the stoichiometric coefficients for the metabolic products	
C_p	Concentration of metabolic product	[mole / liter broth]
$C_{p,in}$	Concentration of inlet metabolic product	[mole / liter feed]
C_s	Concentration of substrate	[mole / liter broth]
$C_{s,in}$	Concentration of inlet substrate	[mole / liter feed]
df	Degree of freedom	
D	Dilution rate	[per hour]
F_{in}	Volumetric flow rate into the bioreactor	[liter feed / hour]
F_{out}	Volumetric flow rate out of the bioreactor	[liter / hour]
$\overline{\overline{F}}$	Variance-covariance matrix	
g_{ji}	Stoichiometric coefficient for the i^{th} intracellular metabolite in the j^{th} reaction	
$\overline{\overline{G}}$	Matrix containing the stoichiometric coefficients for the intracellular metabolites	
$\overline{\overline{G}}_c$	Matrix containing the stoichiometric coefficients for the intracellular metabolites in reactions for which fluxes are not measured	
$\overline{\overline{G}}_m$	Matrix containing the stoichiometric coefficients for the intracellular metabolites in reactions for which fluxes are not measured	
$h_{s,i}$	Carbon content in the i^{th} substrate	[C-mole / mole]
$h_{p,i}$	Carbon content in the i^{th} metabolic product	[C-mole / mole]
I	The number of constraints	
$\overline{\overline{I}}$	Identity matrix	
J	The number of unknown reaction rates	
K	The number of intracellular metabolites considered in the analysis	
M	The number of metabolic products considered in the analysis	
N	The number of substrates considered in the analysis	
P_i	The i^{th} metabolic product	
$\overline{\overline{P}}$	Variance-covariance matrix of residuals	
Q	The number of macromolecular pools considered in the analysis	
r	Specific rate	[mole / gram residual-cell weight / hour]
r_d	Specific rate of cell death	[gram / gram residual-cell weight / hour]

Symbol	Definition	Unit
$r_{macro,i}$	Specific rate of formation of the i^{th} macromolecular pool	[gram / gram residual-cell weight / hour]
$r_{met,i}$	Specific rate of formation of the i^{th} intracellular metabolite	[mole / gram residual-cell weight / hour]
$r_{p,i}$	Specific product formation rate of the i^{th} product	[mole / gram residual-cell weight / hour]
$r_{s,i}$	Specific substrate uptake rate of the i^{th} substrate	[mole / gram residual-cell weight / hour]
r_x	Specific rate of viable cell growth	[gram / gram residual-cell weight / hour]
$\bar{r}_{macro}$	Vector containing the specific rates of macromolecular formation	[gram / gram residual-cell weight / hour]
$\bar{r}_{met}$	Vector containing the specific rates of intracellular metabolite formation	[mole / gram residual-cell weight / hour]
$\bar{r}_p$	Vector containing the specific rates of metabolic product formation	[mole / gram residual-cell weight / hour]
$\bar{r}_s$	Vector containing the specific rates of substrate uptake	[mole / gram residual-cell weight / hour]
S_i	The i^{th} substrate	
t	Time	[hour]
$\bar{\bar{T}}$	Matrix containing stoichiometric coefficients	
v_j	Specific rate of the j^{th} reaction	[mole / gram residual-cell weight / hour]
$\bar{v}$	Vector of reaction rates	[mole / gram residual-cell weight / hour]
$\bar{v}_c$	Vector of non-measured reaction rates	[mole / gram residual-cell weight / hour]
$\bar{v}_m$	Vector of measured reaction rates	[mole / gram residual-cell weight / hour]
V	Volume of the culture broth	[liter broth]
X_d	Concentration of non-viable cell	[gram / liter broth]
$X_{macro,i}$	Concentration of the i^{th} macromolecular pool	[gram / gram residual-cell weight]

Symbol	Definition	Unit
$X_{met,i}$	Concentration of the i^{th} intracellular metabolite	[gram / gram residual-cell weight]
X_v	Concentration of viable cell	[gram / liter broth]
$Y_{i/j}$	Yield coefficient	[mole *i* / mole *j*]

Greek symbols

Symbol	Definition	Unit
α_{ji}	Stoichiometric coefficient for the i^{th} substrate in the j^{th} reaction	
β_{ji}	Stoichiometric coefficient for the i^{th} metabolic product in the j^{th} reaction	
$\bar{\delta}$	Vector of measurement errors	
$\bar{\varepsilon}$	Vector of residuals	
χ_{ji}	Stoichiometric coefficient for the i^{th} macromolecular biomass constituent in the j^{th} reaction	
$\bar{\bar{\Gamma}}$	Matrix containing the stoichiometric coefficients for the macromolecular pools	
μ	Specific growth rate	[per hour]

Abbreviations

3HB	3-hydroxybutyrate monomeric unit
3HV	3-hydroxyvalerate monomeric unit
ADP	Adenosine 5-diphosphate
ATP	Adenosine 5-triphosphate
ED	Entner-Doudoroff pathway
EMP	Embden-Meyerhof-Parnas pathway
FAD	Flavin adenine dinucleotide, oxidized
$FADH_2$	Flavin adenine dinucleotide, reduced
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PHA	Polyhydroxyalkanoate
PP	Pentose phosphate pathway
TCA	Tricarboxylic acid cycle pathway

CHAPTER 1
INTRODUCTION

1.1 General prospect
1.2 Thesis objectives
1.3 Scope of study
1.4 Expected benefits
1.5 Thesis approach

1.1 General prospect

Nowadays humanity has confronted with varieties of threatening catastrophic disasters; for instances, famine, flooding, aridity, and so on. Due to limited resources, most of those disasters have been caused by the irresponsible actions of humanity himself. Providing that good management of valuable resources is recognized accompanied by accomplishment of appropriate technology, those perilous consequences will not occur.

Among those already-occurred menacing consequences, overwhelmed waste has been considered as one of the most serious problems in the modern age. Tons of either municipal or industrial wastes have been daily generated and caused either directly or indirectly great impact on the global environment.

Specifically, plastic waste, which has caused increasing problems in our modern lives, has been reported to be one of the most anxiously critical constituents raising a number of environmental and human health concerns. Most plastic materials, for instance, are not biodegradable and are derived from nonrenewable resources. The properties of durability and strength that make these materials so useful also ensure their persistence in the environment and complicate their disposal. In addition, the synthesis of some polymeric materials involves the use of toxic compounds or the generation of toxic by-products. [Poillon, 1993]

Alarmingly, illustrated in *Figure 1.1-1* is the statistical plastic generation and recovery in the United States of America, one of the most leading-technology countries in the world, for the past forty-five years.

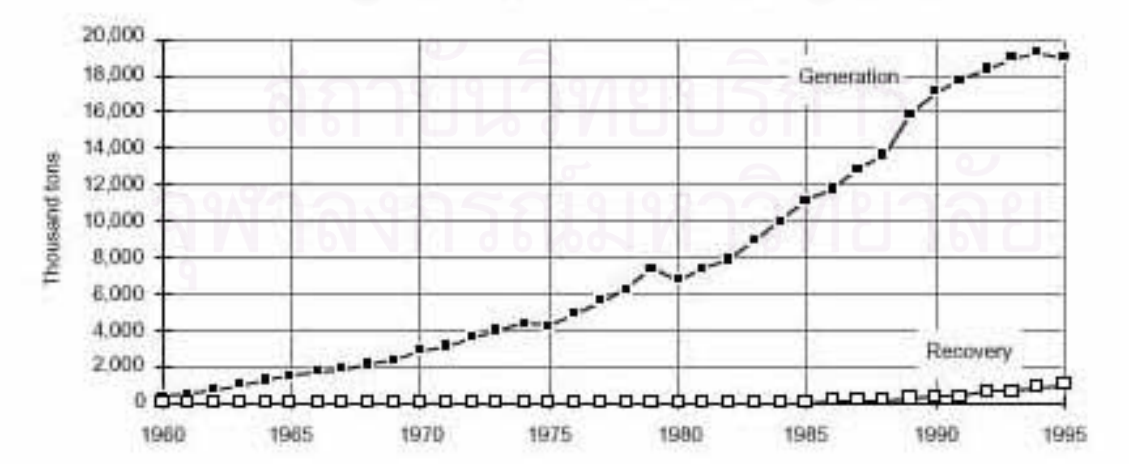
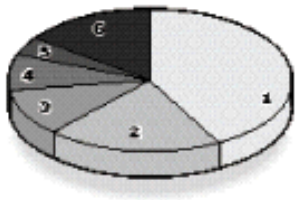


Figure 1.1-1 **: Plastic generation and recovery in the United States of America from 1960 to 1995.** [http://www.epa.gov]

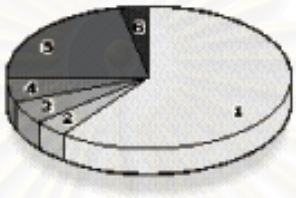
Polyethylene (PE), one of the most well-known petrochemical-based plastic, possess undesirable characteristics of being based entirely on fossil fuels like oil, requiring roughly 2.2 kilograms of fossil fuels per 1 kilogram of polyethylene generated, and not being recyclable to fulfill its original functions. Additionally, oil demands of polyethylene exacerbate fossil fuel pollution of both air and water. This means that polyethylene production has been regarded unsustainable since it was first introduced in 1933. [http://www.geocities.com/Ratspaw_Unlimited/PE2PHA.html]

In spite of realizing this silent threat, petrochemical-based plastic has still been in service. Drastically, the plastic consumption all around the world has been increasing. *Figure 1.1-2* illustrates plastic consumption and plastic waste in Western Europe, only a comparable small region in the world in 1994. As well, plastic consumption with respect to the growth of gross domestic product (GDP) of Thailand from 1995 to 2000 is illustrated as *Figure 1.1-3*.



Plastics consumption by industry sector in Western Europe, 1994 (x 1,000 tonnes). Total consumption: 26,260,000 tonnes (source: APME, 1996a)

1. Packaging	11,029
2. Building/Construction	5,252
3. Electrical & electronic	2,889
4. Automotive	2,101
5. Agriculture	1,050
6. Other (furniture, household goods, toys, distribution large industry and other)	3,939



Plastic waste by sector in Western Europe, 1994 (x 1,000 tonnes). Total waste: 17,505,000 tonnes (source: APME, 1996a)

1. Municipal solid waste	10,581
2. Agriculture	661
3. Construction/demolition works	956
4. Automotive	927
5. Distribution and large industry	3,803
6. Electrical and electronics	577
Total plastic waste Western Europe	17,505

***Figure 1.1-2* : Plastic consumption and plastic waste in Western Europe in 1994.** [http://www.epa.gov]

***Figure 1.1-3* : Plastic consumption with respect to the growth of gross domestic product (GDP) of Thailand from 1995 to 2000.**

One of the most promising solutions for those problems is the development of novel biodegradable plastic or usually known as biopolymer. Unlike petrochemical-based plastic, biodegradable plastic is produced from natural and renewable feedstock like carbohydrate from agricultural plant crop as well as carbondioxide and agricultural waste material. This means that biodegradable plastic production is likely to be undoubtedly sustainable.

Among those stated biodegradable plastics, biopolyester has been of substantial popularity. As a basic perception, polyester is defined as polymer formed by the condensation of polyhydric alcohol and polybasic acid. [Sharp, 1985] Likewise, biopolyester is polyester derived from bacterial sources.

There are two well-known types of this biopolymer family; namely, polyhydroxyalkanoate (PHA) and polylactic acid (PLA). Focused on the first popular type, polyhydroxyalkanoate is exclusively based on hydroxyalkanoic acid. Usually this kind of biopolyester is found in the form of 3-hydroxyalkanoic acid (or equivalent common name as β- hydroxyalkanoic acid), but 4-hydroxyalkanoic acid (or equivalent common name as γ-hydroxyalkanoic acid) is also realized. [Hocking and Marchessault, 1994] Summary of the most frequently-asked questions with the corresponding answers is illustrated in *Table 1.1-1*.

As for the biosynthesis of polyhydroxyalkanoate, diversities of organism have been reported as the potential producers of this type of novel biodegradable polymer, ranging from ordinary classes of prokaryotes like bacteria through higher classes of eukariotes like yeast. Curiously, development of recombinant microorganism and transgenic plants are also in progress.

Known as P(3HB), poly(3-hydroxybutyrate) or poly(β-hydroxybutyrate) is one of the most simplest and most common members of the polyhydroxyalkanoate family. As formerly stated, this kind of biopolyester has been concerned as the potential sustainable development. Illustrated in *Figure 1.1-4*, material cycle reveals the ideal closed-loop material recycle rendering no eternal accumulation of after-use waste.

Well known as P(3HB-*co*-3HV), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) is another one of this typical worldwide biopolymer. Superior to poly(3-hydroxybutyrate) in its mechanical strength, flexibility, and processability, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) is dramatically of great interest in realistic application. Higher characteristics and performance of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) has led lots of researchers and manufacturers undergoing their projects through the midst of mysteries how to obtain the desired novel product from the limited resources in the optimal way. The commercial pioneers questing for that ideality are listed in *Table 1.1-2*.

Table 1.1-1 **: Summary of the most frequently-asked questions with the corresponding answers.** [http://www.metabolix.com]

Question	Answer
What is PHA?	PHA stands for polyhydroxyalkanoate -- the chemical term for a naturally occurring form of polyester. The first PHA was identified by the French microbiologist Maurice Lemoigne in 1925. Since then, over one hundred different PHAs have been described in the scientific literature.
What are PHAs useful for?	There are many different types of PHAs produced by natural and recombinant organisms. PHAs may range from stiff thermoplastics to more flexible, rubbery materials. In addition, some technique has the capability of blending and compounding PHAs with other materials to achieve tailored properties. PHAs are generally suitable for extrusion, injection moulding, as well as film and coating applications. PHAs may also be suitable for biomedical use, for example in long-term drug delivery or orthopedic repair.
What are some of the advantages of PHAs over conventional plastics?	Unlike conventional plastics, PHAs are produced from renewable resources – sugars in the case of a fermentation process; carbon dioxide and sunlight in the case of transgenic plant crops. PHAs are biodegraded by a wide range of microorganisms, and they can also be managed by recycling or hydrolysis. In addition, the PHAs have a number of performance attributes (e.g. excellent barrier properties, low temperature thermolysis) which set them apart from conventional materials.
If PHAs are so good, why aren't they in more widespread use?	PHAs have been produced in industrial quantities by fermentation for a number of years. PHAs have been used commercially for cosmetic bottles, paper coatings, and medical implants. The costs of PHAs have been higher than for petroleum-based plastics, limiting their application to certain niche markets. However, genetic technology now allows the production of PHAs at more competitive prices. In the future, transgenic plant crops utilizing some patented genes will bring costs down further.

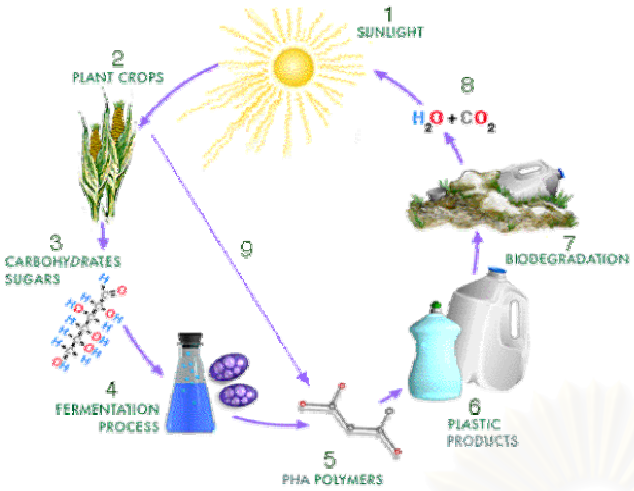


Figure 1.1-4 **: Idealistic material cycle of the polyhydroxyalkanoate family.** [http://www.metabolix.com]

Table 1.1-2 **: Large commercial manufacturers of typical biopolymers and applications of the corresponding biopolymers.** [Kim, I. *et al*, 1998]

Trade name	Type	Manufacturer	Applications
Biopol	P(3HB-*co*-HV)	Monsanto	- Cosmetics packaging - Food-service packaging and utensils - Agricultural containers and films - Fishing net - Garbage bags
Heplon	PLA	Chronopol	- Fibers, non-wovens, blown, cast and oriented films - Injection-molded, thermoformed and blow-molded films - Multi-layer extrusions - Coatings and adhesives - Rigid and flexible foams
EcoPLA	PLA	Cargill	- Leaf bags - Trash-bin liners - Disposable food-service utensils and containers - Agricultural films - Golf tees
Lacea	PLA	Mitsui	- Foams - Blown bottles - Films - Coatings - Containers - Tableware
Cargill-Dow PLA	PLA	Cargill Dow	- Cast films - Non-woven hygiene products - Fibers - Bicomponent materials

Theoretically, conventional plastics may be substituted by biopolymers including poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in many applications. In practice, substitution is not always either feasible, wanted or the most lucrative way to use biopolymers. Besides technical development which is needed for some applications, the applications have to be economically feasible within a reasonable term. The economic feasibility depends on the investments needed for material and product development and the added value of the biopolymer in the application. The added value is of great importance because the prices of biopolymers are higher than those of conventional plastics.
[http://www.proterra.nl/biopolymers.html]

To achieve the consistency of the so-called 3E principles; engineering prospect, economic prospect, and environmental prospect, the application of biotechnology and genetic engineering has been one of the most promising solutions of those difficulties. There are two main techniques involving that application of both biotechnology and genetic engineering; namely, classical mutagenesis and recombinant deoxyribonucleic acid (DNA) technology.

Metabolic engineering, an interesting field of newly-emerging subject, has been proposed as the potential tool to systematically merge the advantage of both biotechnology and genetic engineering. First suggested by Bailey in 1991, metabolic engineering has been extensively one of the most frequently referred issues in modern research activity focusing on biological and biochemical engineering, cell physiology, and applied microbiology. Furthermore, it is now well-developed to be a central topic in biochemical engineering courses at both graduates and undergraduates of the Massachusetts Institute of Technology (MIT), USA and the Technical University of Denmark (DTU), Denmark. [Stephanopoulos *et al*, 1998]

As for its definition, metabolic engineering is considered as directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology. [Bailey, 1991 and Stephanopoulos *et al*, 1998]

In conclusion, this research will focus on application of the first stage of the analytical side of metabolic engineering, metabolic flux analysis, with poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis by *Ralstonia eutropha* NCIMB 11599 in the fed-batch cultivation. Probably, concept of metabolic pathway analysis may be applied in supporting any ideas in the experimental analysis and discussion. It can be noticed that, so far investigated, this is the first research where metabolic flux analysis has been applied to investigate the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthetic pathways in microbial cells. Due to its comparatively fast-growing and high-biopolymer-content-yielding prominence, this bacterial strain is selected for the studied biosynthesis. The desired target for metabolic engineering is an improvement of molar fraction of 3-hydroxyvalerate monomeric unit in the copolymer which can be achieved through the understanding of flux distributions when two key parameters are varied. These parameters are the ratio of present carbon to nitrogen and the molar percentage of glucose in the reaction medium. Hopefully, the suggested strategies to enhance the molar 3-hydroxyvalerate monomeric unit in the copolymer could make poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) much more value-added.

1.2 Thesis objectives

To apply metabolic flux analysis for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis by *Ralstonia eutropha* NCIMB 11599 to serve the following objectives.

1. To identify metabolic pathways in the proposed metabolic networks.
2. To determine maximal theoretical molar fraction of 3-hydroxyvalerate monomeric unit in the copolymer.
3. To identify principal nodes and possible bottlenecks in the proposed metabolic networks.
4. To suggest rational modifications for metabolic engineering.

1.3 Scope of study

As for the relevant scope of study, metabolic flux analysis of poly(3-hydroxybutyrate-co-3-hydoxyvalerate) biosynthesis in *Ralstonia eutropha* NCIMB 11599 is successfully carried out by the application of MATLAB program. The time-series concentration profiles of extracellular substrates and products as input data are taken from the experimental data of Kasipar (2002) in which two operating parameters were varied. These two parameters are molar carbon-to-nitrogen ratio and molar fraction of propionic acid, which are varied from 4-200 and 0-100%, respectively.

1.4 Expected benefits

1. Able to elucidate possible bottleneck steps in the studied metabolic network.
2. Able to suggest information and hints for further step of rational modifications of the studied metabolic network.
3. Able to be such contribution to further development of biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), one of the most novel biodegradable plastic, which may lead to the more feasible industrial production in the future.

1.5 Thesis approach

To accomplish this thesis, there are seven main stages as shown in *Figure 1.5-1*; namely, development of biochemically-feasible networks, development of mathematically-feasible networks, development of thermodynamically-feasible networks, determination of maximal theoretical 3HV molar fraction, comparison of theoretical with experimental values, identification of principal nodes and possible bottlenecks, and suggestion of strategies for metabolic engineering. Details of each stage will be covered in chapter 4 and chapter 5 of this thesis.

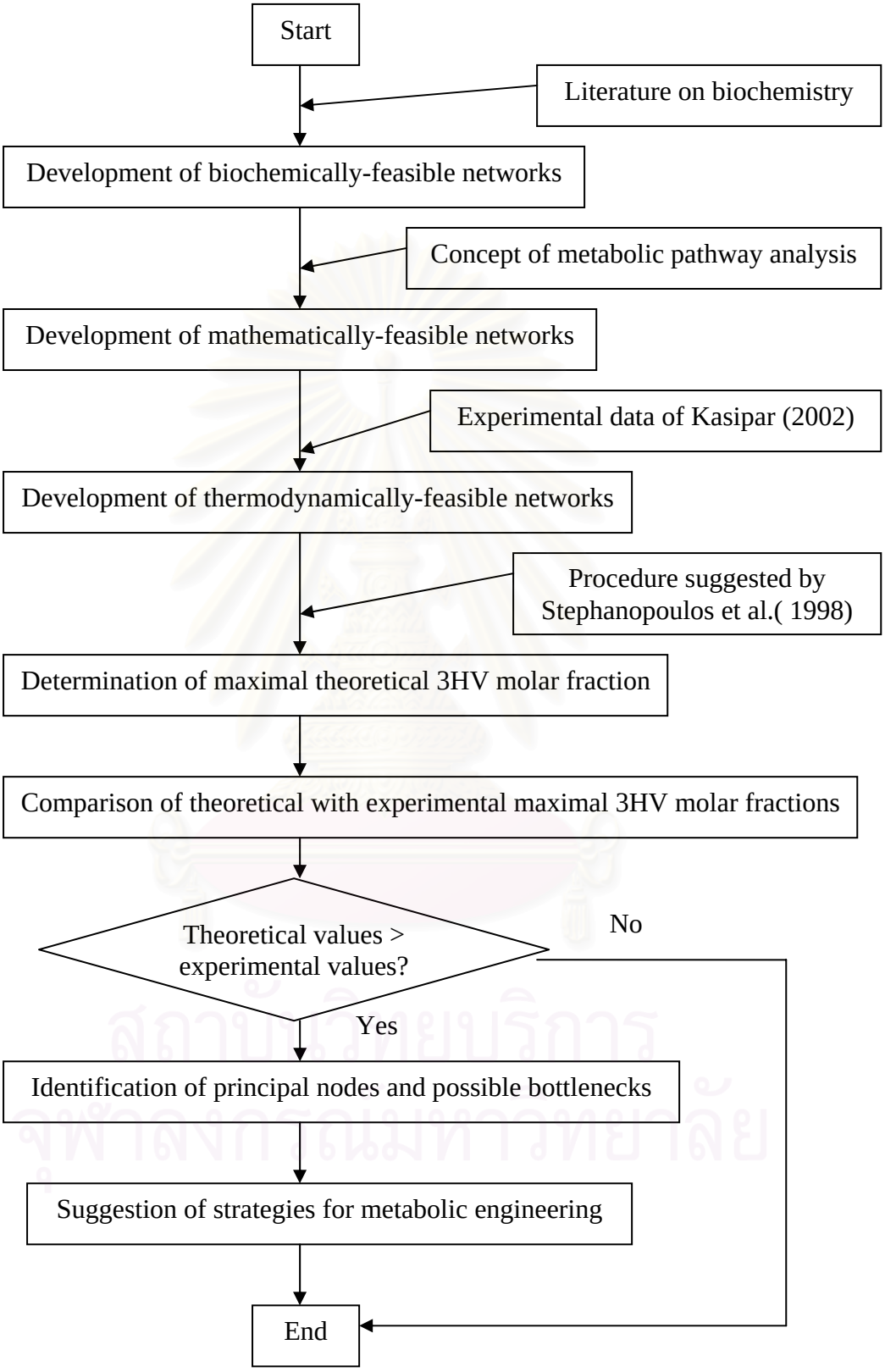


Figure 1.5-1: Thesis approach.

CHAPTER 2
LITERATURE REVIEW

2.1 General features of polyhydroxyalkanoate
2.2 Cellular physiology and biochemistry of polyhydroxyalkanoate-biosynthesizing microorganism
2.3 Biosynthesis of polyhydroxyalkanoate
2.4 Metabolic engineering

Collectively, researches on polyhydroxyalkanoate biosynthesis can be categorized into six main issues; namely, general features of polyhydroxyalkanoate, cellular physiology and biochemistry of polyhydroxyalkanoate-biosynthesizing bacteria, biosynthesis of polyhydroxyalkanoate, recovery of polyhydroxyalkanoate, processing and application of polyhydroxyalkanoate, and environmental impact of polyhydroxyalkanoate.

Among those issues, only three key issues will be further covered in details due to their relevance to this research; namely, general features of polyhydroxyalkanoate, cellular physiology and biochemistry of polyhydroxyalkanoate-biosynthesizing bacteria, and biosynthesis of polyhydroxyalkanoate.

As for researches on metabolic engineering, several studies can be categorized into two overview aspects; approaching-oriented and targeting-oriented point of view. Greater detail will be further covered according to the relevant aspects

2.1 General features of polyhydroxyalkanoate

First mentioned in the microbiological literature, poly(3-hydroxybutyrate) was initially discovered as the first member of polyhydroxyalkanoate family in 1901. [Beijerinck, 1901 stated in Hocking and Marchessault, 1994] Since then, much more detailed studies on polyhydroxyalkanoates have been extensively carried out.

As for general features of polyhydroxyalkanoate, there are mainly three constitutional aspects totally involved; namely, chemical structure, property characteristics, and biodegradation consideration. Due to the specified scope of study, only chemical structure and property characteristics of polyhydroxyalkanoate will be further covered leaving the detailed discussion of the latter barren.

2.1.1 Chemical structure

In 1925, Lemoigne reported that granule-like inclusions could be observed in the cytoplasmic fluid of bacteria. Like ordinary lipids, the inclusions were not ether soluble. Verified by microscopic observations, saponification and acid numbers, autolysis, solubility and melting point variation with molecular size, and optical activity, he showed that this material was a polyester having the empirical formula of $(C_4H_6O_2)_n$. In addition, differences in melting point observed in two isolated fractions were also remarked by different degree of polymerization. [Lemoigne, 1925a; Lemoigne, 1925b; Lemoigne, 1926; and Lemoigne, 1927 : all stated in Hocking and Marchessault, 1994]

In 1952, Kepes and Péaud Lenoël (1952) [stated in Hocking and Marchessault, 1994] observed that both polyester fractions isolated by Lemoigne were the hydrolysis products of a high molecular weight linear polyester with a melting temperature of 180 degree Celsius. The material was likely to bear functional groups of a carboxylic acid at one end and an alcohol at

the other end. Subsequently, the biopolyester has been well-known as poly(3-hydroxybutyrate), which is abbreviated as P(3HB).

With a modern knowledge on chemistry, the biopolyester can be decoded for the representative chemical structure. Primarily, the biopolyester is linearly head-to-tail polymer consisting of thousands of repeating monomeric units in the form of hydroxy fatty acid.

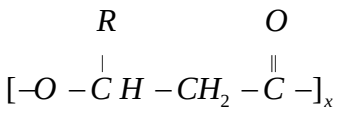
At the same β position, the third carbon, an alkyl group which can vary from methyl to tridecyl is positioned. Generally, the alkyl group can be in the form of saturated, unsaturated, aromatic, halogenated, epoxidized, and branched monomer. [Abe *et al.*, 1990; Choi, M.H. and Yoon, 1994; Curley *et al.*, 1996; Doi and Abe, 1990; Fritzsche *et al.*, 1990a; Fritzsche *et al.*, 1990b; Fritzsche *et al.*, 1990c; Hazer *et al.*, 1994; Kim, Y.B. *et al.*, 1991; Kim, Y.B., 1992; Lageveen *et al.*, 1988; and Song and Yoon, 1996 : all stated in Madison and Huisman, 1999]

As well, an alkyl group can be incorporated into the other carbon position to form a 4-hydroxyalkanoate, 5-hydroxyalkanoate, and 6-hydroxyalkanoate. [Eggink *et al.*, 1995; Kunioka *et al.*, 1989; Valentin *et al.*, 1992; Valentin *et al.*, 1994; and Valentin *et al.*, 1996 : all stated in Madison and Huisman, 1999]

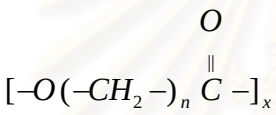
Appealingly, the repeating monomeric units can reflect such characteristics of the biopolyester; for examples, physical properties, mechanical properties, and biodegradation characteristics. Thus, it is urged that the biopolyester be categorized according to the representation of repeating monomeric unit.

In accordance with the type of repeating monomeric unit, there are mainly two classes of polyhydroxyalkanoate; namely, short-side-chain and medium-side-chain polyhydroxyalkanoates. A short-side-chain polyhydroxyalkanoate consists of an organic ester derived from certain volatile acid. While a comparatively longer chain of fatty ester, say six to sixteen carbon-containing molecule, can be recognized as a constituent of a medium-side-chain polyhydroxyalkanoate. Typical chemical structure of polyhydroxyalkanoate can be consulted from *Figure 2.1-1*.

In addition, the polyhydroxyalkanoate can be recalled as a homopolymer or a copolymer. The biopolyester that comprises merely one compound as a repeating monomeric unit is categorized as a homopolymer. On the other hand, the biopolyester is named as a copolymer if it is composed of more than one compound as a repeating unit. Specifically, among nearly a hundred members of polyhydroxyalkanoate family, one of the most renowned biocopolyester is poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), which is universally abridged as P(3HB-*co*-3HV). Typical chemical structure of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) is shown as *Figure 2.1-2*.

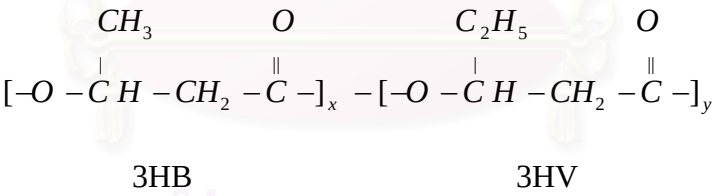


R = hydrogen	3-hydroxypropionate	(3HP)
R = methyl	3-hydroxybutyrate	(3HB)
R = ethyl	3-hydroxyvalerate	(3HV)
R = propyl	3-hydroxycaproate	(3HC)
R = butyl	3-hydroxyheptanoate	(3HH)
R = pentyl	3-hydroxyoctanoate	(3HO)
R = hexyl	3-hydroxynonanoate	(3HN)
R = heptyl	3-hydroxydecanoate	(3HD)
R = octyl	3-hydroxyundecanoate	(3HUD)
R = nonyl	3-hydroxydodecanoate	(3HDD)



n = 3	4-hydroxybutyrate	(4HB)
n = 4	5-hydroxyvalerate	(5HV)

Figure 2.1-1 **: Typical chemical structure of polyhydroxyalkanoate.** [Stephanopoulos *et al.*, 1998]



3HB 3HV

Figure 2.1-2 **: Typical chemical structure of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).**

2.1.2 Property characteristics

As for property characteristics of polyhydroxyalkanoate, data on molecular weight and certain physical properties of poly(3-hydroxybutyrate) were first reported by Williamson and Wilkinson (1958) [stated in Hocking and Marchessault, 1994].

Concerning the succeeding studies on physical properties, molecular weight distribution and melting temperature was of popularity in the pioneering age. A wide range of molecular weights and melting points were reported for poly(3-hydroxybutyrate) depending upon the method of isolation and the polyester-biosynthesizing bacterial strain. [Lundgren *et al.*, 1965; and Okamura, 1967 : all stated in Hocking and Marchessault, 1994] Particularly, under mild conditions like solvent extraction and direct isolation of native granules, high-molecular-weight polymers can be obtained with weighted-average molecular weights ranging from a few hundred thousand to a million or more. [Merrick, 1978; Baptist, 1962a; Baptist, 1962b; and Marchessault *et al.*, 1970 : all stated in Hocking and Marchessault, 1994]

Within the cell, poly(3-hydroxybutyrate) exists in an amorphous fluid state. However, after an extraction, the biopolyester becomes highly crystalline and hence stiff but brittle material. [Doi, 1995 stated in Hocking and Marchessault, 1994] Its inferiority of certain mechanical properties limits its range of processing and application. Arousingly, development of the other member of polyhydroxyalkanoate including poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) has been attempted for a better performance plastic.

Tabulated in *Table 2.1-1* are the typical property characteristics of a conventional poly(3-hydroxybutyrate). As formerly stated, certain property characteristics are tentative to change in keeping with the characteristics of the repeating monomeric unit. As an example, effects of an incorporation of 3-hydroxyvalerate monomeric unit to form a semi-crystalline copolymer on certain mechanical properties are summarized as *Table 2.1-2*.

Table 2.1-1 **: Typical property characteristics of poly(3-hydroxybutyrate)** [http://www.goodfellow.com/scripts/web.wl?MGWLPN=MNT]

Property category	**Property description**	**Property characteristics**
Physical property	Density	1.25 g/cm^3
	Resistance to ultraviolet	Fair
Thermal property	Upper working temperature	95 °C
Mechanical property	Izod impact strength	35-60 J/m
	Young's modulus	3.5 G Pa
	Tensile strength	40 M Pa
Optical property	Structural chirality	D- (or R) configuration
Electrical property	Dielectric constant at 1 Mega hertz	3.0
	Volume resistivity	10^{16} Ohm.cm
Chemical resistance	Dilute acids	Fair
	Alcohols	Fair
	Alkalis	Poor
	Greases and oils	Good

Table 2.1-2 **: Typical mechanical properties of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) according to the corresponding mole fraction of the 3-hydroxyvalerate unit.** [Holmes, 1988 stated in Hocking and Marchessault, 1994]

Mole fraction of HV unit (%)	Melting temperature (°C)	Glass transition temperature (°C)	Young's modulus (G Pa)	Tensile strength (M Pa)	*Notched-Izod impact strength (J/m)
0	179	10	3.5	40	50
3	170	8	2.9	38	60
9	162	6	1.9	37	95
14	150	4	1.5	35	120
20	145	-1	1.2	32	200
25	137	-6	0.7	30	400

Note : * With 1 millimeter radius notch.

In addition, as previously mentioned in Chapter 1, the polyhydroxyalkanoate is likely to be a sustainable thermoplastic that can be replaced the conventional petroleum-based plastic due to its resemblance of certain property characteristics. The superiority of the polyhydroxyalkanoate is its biodegradability and biocompatibility. On the other hand, its inferiority is its relatively higher production cost. Consequently, the use of polyhydroxyalkanoate is nowadays limited to a specialty plastic not a commodity plastic. Hereby, a comparison between polyhydroxyalkanoates and polypropylene is introduced as in *Table 2.1-3*.

Table 2.1-3 **: Comparison of certain property characteristics between those of two biopolyester and polypropylene.**

Property description	P(3HB)	P(3HB-*co*-3HV)	Polypropylene
Density (g/cm^3)	1.250	N/A	0.905
Crystallinity (%)	80	56	70
Molecular weight (Dalton)	$5 * 10^5$	N/A	$2 * 10^5$
Crystalline melting temperature (°C)	175	145	176
Glass transition temperature (°C)	-4	-1	-10
Flexural modulus (G Pa)	4.0	1.2	1.7
Tensile strength (M Pa)	40	32	38
Extension to break (%)	6	50	400
Ultraviolet resistance	Good	N/A	Poor
Solvent resistance	Poor	N/A	Good

Note :

- N/A means data not available from the stated reference.
- Data of poly(3-hydroxybutyrate) and polypropylene are referred to those reported by Howells (1982) [stated in Hocking and Marchessault, 1994].
- Data of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) are referred to those of 20-percent 3-hydroxyvalerate unit containing biopolyester reported by Doi (1990).

2.2 Cellular physiology and biochemistry of polyhydroxyalkanoate-biosynthesizing microorganism

Focus on a polyhydroxyalkanoate-biosynthesizing microorganism, two main aspects are to be briefly summarized; namely, cellular physiology and cellular biochemistry.

2.2.1 Cellular physiology

As for the physiological aspect, scientists came to the conclusion that bacteria store polyhydroxyalkanoate as a carbon and energy reserve material in a manner like starch and glycogen that were believed to be accumulated in other organisms. [Macrae and Wilkinson, 1958; Doudoroff and Stanier, 1959; and Wilkinson, 1963 : all stated in Hocking and Marchessault, 1994]

Also confirmed by the study of Dawes (1986) [stated in Hocking and Marchessault, 1994], the water-insoluble molecules of poly(3-hydroxybutyrate) exert a negligible intracellular osmotic pressure. As a result, it can be noted that this biopolyester can be served as an ideal reserve material stored in the cells.

As an inclusion granule, the poly(3-hydroxybutyrate) inclusion is normally a spherical body of 0.5 micrometers in diameter. [Baptist, 1962a; Baptist, 1962b; Ellar *et al.*, 1968; and Merrick, 1978 : all stated in Hocking and Marchessault, 1994] Microscopic picture of poly(3-hydroxybutyrate) accumulated inside the microbial cell is shown as *Figure 2.2-1*.

Not only polyhydroxyalkanoate can be observed in the form of inclusion bodies, such short chains of the polymer can be isolated from bacterial membranes. [Reusch and Sadoff, 1983; Reusch *et al.*, 1986; and Reusch and Sadoff; 1988 : all stated in Hocking and Marchessault, 1994] As well, poly(3-hydroxybutyrate) can be isolated from plant and animal tissues. [Reusch, 1989; and Reusch *et al.*, 1992 : all stated in Hocking and Marchessault, 1994] Although small amounts of poly(3-hydroxybutyrate) can be noticed as the other stated forms, the following conclusion is somewhat interesting. Poly(3-hydroxybutyrate) is likely to serve other physiological function other than as a reserve material. [Reusch, 1992 stated in Hocking and Marchessault, 1994]

Under a regular growth, one to thirty percents of the dry-cell weight as poly(3-hydroxybutyrate) can be accumulated within the bacterial cell. However, there have been lots of researches reporting that up to ninety percents of the dry-cell weight as accumulated poly(3-hydroxybutyrate) can be attained under certain controlled fermentation conditions with an imbalanced nutrition. [Baptist, 1962a; Baptist, 1962b; Dawes and Senior, 1973; Ward *et al.*, 1977; and Müller and Seebach, 1993 : all stated in Hocking and Marchessault, 1994]

It was first suggested by Macrae and Wilkinson (1958) [stated in Hocking and Marchessault, 1994] that the accumulation of poly(3-hydroxybutyrate) can be increased by a limitation of nitrogen in the growth medium. Following that suggestion, limitation of certain nutrients has been extensively employed for development of a more efficient biopolyester production. For instances, nitrogen limitation [Schlegel *et al.*, 1961; Dawes and Senior, 1973; Emeruwa and Hawirko, 1973], oxygen limitation [Ward *et al.*, 1977], and mineral ion limitation [Suzuki *et al.*, 1986a].

To explain those observations, cellular physiology is to be clarified. When nutrient supply is imbalanced, it is advantageous for bacteria to store excess nutrient as an intracellular reserve. This can be triggered by limitation of nitrogen, phosphorus, oxygen,

potassium, or sulfur while maintaining the carbon source in excess. [Anderson and Dawes, 1990]

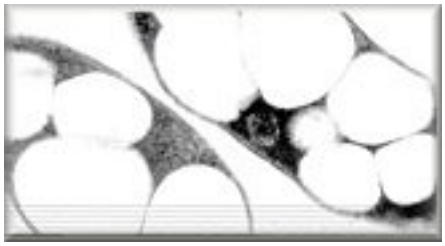


Figure 2.2-1 : **Microscopic picture of poly(3-hydroxybutyrate) accumulated within the microbial cell.** [http://www.metabolix.com]

The biopolyester inclusion can be biosynthesized by various gram-positive and gram-negative bacteria. The most commonly referred species are in the genus of *Alcaligenes*, *Methylobacterium*, *Pseudomonas*, *Sphaerotilus*, *Agrobacterium*, *Rhodobacter*, *Actinobacillus*, and *Azotobacter*.

It is wise to categorize those bacteria into two groups; a growth-associated producer and a non-growth-associated producer. A growth-associated producer does not require any nutrient limitations during the biopolyester synthesis. While a non-growth-associated producer is strictly dealing with certain nutrient limitations for efficient biosynthesis of biopolyester.

Ralstonia eutropha, which is formerly known as *Alcaligenes eutrophus*, can be classified as a non-growth-associated producer. Certain cellular characteristics of the genus *Alcaligenes* are summarized in *Table 2.2-1*.

Specifically, *Ralstonia eutropha*, the strain of interest, is a short rod, 0.5 micrometer in diameter and 1.8-2.6 micrometers in length. It is sluggishly motile by means of 1-4 peritrichous flagella. Optimum temperature for cell growth is about 30 degree Celsius. Colonies are opaque with white or cream in color. After several days, the colonies may become brown. As a sole carbon source, a variety of organic compounds can be supplemented; for instances, glucose, fructose, testosterone, phenol, and benzoate. However, it cannot utilize ethanol, glycerol, mannitol, pentoses, disaccharides, starch, gelatin, D-tryptophan, and acetamide. [Buchanan *et al.*, 1974]

Because of its dominance in fast growing and capability of accumulating a high content of biopolyester, *Ralstonia eutropha* has been one of the most preponderant strains used in the study on a short-side-chain polyhydroxyalkanoate biosynthesis.

Table 2.2-1 **: Certain cellular characteristics of the genus *Alcaligenes*. [Buchanan *et al*., 1974]**

Cellular characteristics	**Description**
Gram reaction	Negative
Cell shape	Short rods to coccoid
Cell diameter (micrometer)	0.5-1.0
Motility in liquid media	+
Flagellar arrangement	Lateral or subpolar
Yellow carotenoid cellular pigments	-
NO_3^- reduced to NO_2^-	+
Anaerobic growth with NO_3^-	+
Anaerobic growth with NO_2^-	-
H2 chemolithotrophy	+
Gelatin hydrolysis	-
Utilization of 3-hydroxybutyrate	+
Can use methanol as a carbon source	-
Carbon sources for growth :	
D-glucose	M
L-arabinose	-
D-xylose	-
D-fructose	+
D-mannitol	-
D-mannose	-
D-gluconate	+
Acetate	+
Adipate	+
Pimelate	+
Sebacate	+
Suberate	+
meso-tartrate	+
Itaconate	+
Genus includes pathogens, frank, or opportunistic :	
Human and/or animals	-
Plants	+

Note : + means 90% or more of strains are positive.
- means 10% or less of strains are positive.
M means mutant growth.

2.2.2 Cellular biochemistry

Intensively, there have been lots of studies on the metabolic pathway for biosynthesis and degradation of polyhydroxyalkanoates. [Merrick and Doudoroff, 1961; Merrick, 1978; Dawes, 1986; Doudoroff and Stanier, 1959; Ritchie and Dawes, 1969; Moskowitz and Merrick, 1969; Ritchie *et al..*, 1971; Senior and Dawes, 1971; Senior and Dawes, 1973; Fukui *et al..*, 1976; Jackson and Dawes, 1976; Stanier *et al..*, 1976; Nishimura *et al..*, 1978; Shuto *et al..*, 1981; Doi *et al..*, 1987; and Haywood *et al..*, 1988 : all stated in Hocking and Marchessault, 1994]

As a review of poly(3-hydroxybutyrate), its building block is acetyl-coenzyme A (acetyl-CoA). The building block can be synthesized from feedstocks like glucose, fructose, sucrose, methanol, acetic acid, and even gaseous mixture of carbondioxide and hydrogen. With the action of three present enzymes, the polymerization can be carried out. Those stated enzymes are 3-ketothiolase, acetoacetyl-coenzyme A reductase, and PHB synthase, respectively. The first enzyme, 3-ketothiolase is capable of condensing two molecules of acetyl-coenzyme A to form acetoacetyl-coenzyme A (acetoacetyl-CoA). Then an NADPH-dependent stereoselective acetoacetyl-coenzyme A reductase will reduce the obtained acetoacetyl-coenzyme A to R-3-hydroxybutyryl-coenzyme A (R-3-hydroxybutyryl-CoA). Finally, the last enzyme, PHB synthase will bind the 3-hydroxyl group of the obtained R-3-hydroxybutyryl-coenzyme A to the carboxyl end of a pre-existing polymeric chain to form an ester bond. Consequently, the polymeric chain will be lengthening by one at a time. [Senior and Dawes, 1971; Senior and Dawes, 1973; Nishimura *et al..*, 1978; and Haywood *et al..*, 1988 : all stated in Hocking and Marchessault, 1994]

It is to be noticed that the proposed biochemistry of poly(3-hydroxybutyrate) was confirmed by the study of Doi *et al..* in 1987 [stated in Hocking and Marchessault, 1994]. The study employed the experiments using ^{13}C-labelled substrates and ^{13}C nuclear magnetic resonance (NMR) spectral analysis of the isolated poly(3-hydroxybutyrate) biosynthesized by *Ralstonia eutropha*.

As for a biochemistry of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis, the following mechanism was proposed with the supporting idea from NMR analysis of ^{13}C-labelled copolyester isolated from *Ralstonia eutropha* H16 in culture media containing [1-^{13}C] propionate, [1-^{13}C] acetate, and [2-^{13}C] acetate. [Doi *et al..*, 1986; and Doi *et al..*, 1987] When propionate is used as the sole carbon source, propionyl-coenzyme A (propionyl-CoA) selectively eliminates the carbonyl carbon turning to be acetyl-coenzyme A. Likewise, 3-hydroxybutyryl-coenzyme A is the condensation product of two molecules of acetyl-coenzyme A. In addition, acetyl-coenzyme A can also react with the obtained propionyl-coenzyme A to form 3-hydroxyvaleryl-coenzyme A (3-hydroxyvaleryl-CoA). Finally, the copolymer can be attained by copolymerization of the obtained 3-hydroxybutyryl-coenzyme A and the obtained 3-hydroxyvaleryl-coenzyme A by the action of PHB polymerase. It can be observed that the ratio of 3HV to 3HB units in the copolyester increased with the concentration of propionate in the culture medium suggesting that the ratio of 3-hydroxyvaleryl-coenzyme A to 3-hydroxybutyryl-coenzyme A increased with the concentration of propionyl-coenzyme A in the cell. When both acetate and propionate are used as carbon sources, acetyl-coenzyme A in the cell can be synthesized from either substrate; as a result, the decreasing proportion of 3HV units in the copolymer can be observed.

Pictorial diagram of metabolic pathway for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) is depicted in *Figure 2.2-2*.

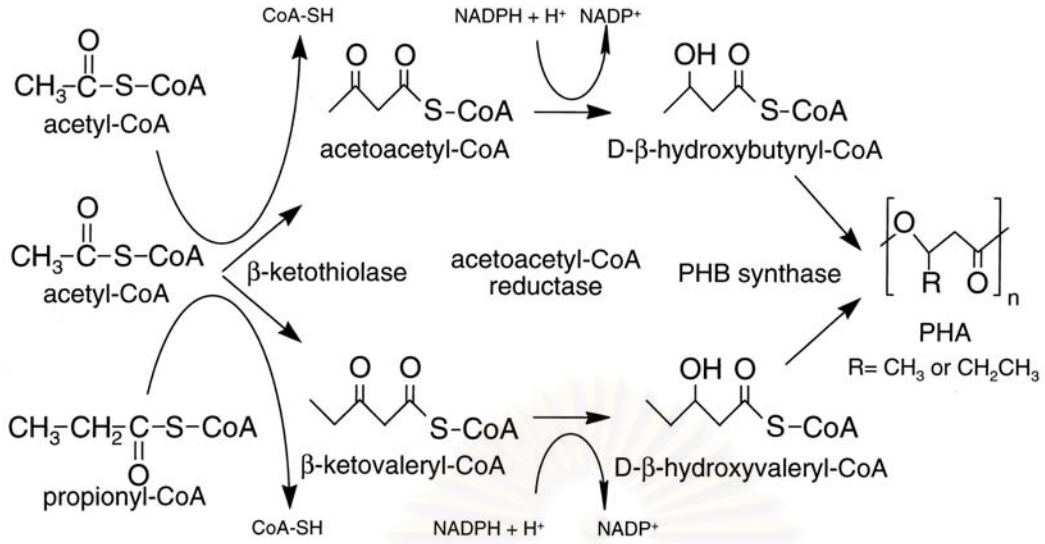


Figure 2.2-2 **: Schematic diagram of synthetic pathway of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).** [Slater *et al.*, 1998]

Moving to a bigger picture of biosynthesis of copolyester, every single chain of the polymer is to be stored inside the cell as granules. As a basic concept of polymeric granule formation, Ellar *et al.* (1968) [stated in Hocking and Marchessault, 1994] reported that there were several thousands of molecules of poly(3-hydroxybutyrate) per granule. Additionally, as a whole picture, there were approximately constant number of eighteen thousands polymerase molecules per cell throughout the accumulation stage of poly(3-hydroxybutyrate) [Kawaguchi and Doi, 1992; and Doi *et al.*, 1992 : all stated in Hocking and Marchessault, 1994] Moreover, the model for polymeric granule formation based on three steps of chain initiation, propagation and transfer, and chain cessation was proposed to account for the observed increased number of polymeric chains during the accumulation stage of poly(3-hydroxybutyrate).

2.3 Biosynthesis of polyhydroxyalkanoate

The literature on the biosynthesis of polyhydroxyalkanoate by *Ralstonia eutropha* is somewhat confusing due to the difference of bacterial strains that were in use. Practically, the following strains have been selected due to their most extensively studied; an original producer H16 (ATCC 17699), a glucose-utilizing mutant NCIMB 11599, a chemolithotroph ATCC 17697^{T}, a versatile copolymer producer SH-69, and a natural isolate *Alcaligenes* sp. Strain AK201. [Madison and Huisman, 1999]

As a result, reviews for the stated strains will be further covered in details. However, such reviews for other strains and other genus might be informative to be compared and be occasionally discussed in a concise form. The mentioned neighboring genus is *Alcaligenes latus*, a growth-associated producer. Recombinant *Escherichia coli* genetically engineered with plasmid from *Ralstonia eutropha* is also included.

2.3.1 Mode of operation

So far biosynthesis of polyhydroxyalkanoate has been achieved by three modes of operation; batch, fed-batch, and continuous mode. Summarized in *Table 2.3-1* are certain suggestive references according to the employed mode of operation.

Table 2.3-1 **: Certain suggestive references according to the employed mode of operation.**

Batch cultivation	**Fed-batch cultivation**	**Continuous cultivation**
Akiyama and Doi, 1993 Beaulieu *et al.*, 1995 Grothe *et al.*, 1999 Lee, I.Y. *et al.*, 1993 Tanaka *et al.*, 1995	Kim. B.S. *et al.*, 1994 Ryu *et al.*, 1997 Shi *et al.*, 1997 Sugimoto *et al.*, 1999 Vingvon, 1996 Yamane *et al.*, 1996b	Gostomski and Bungay, 1996 Henderson and Jones, 1997 Koyama and Doi, 1995

In batch cultivation, both cell growth and biopolyester formation are examined in the same medium. Usually, this cultivation technique has been employed as the first stage of the experimentation to investigate certain culture characteristics during the fermentation. Typically, timing of harvest can be suggested through the obtained time profile of the microbial lag, exponential, and stationary phases. As well, the most crucial microbial characteristics can be estimated through a development of kinetic modeling. However, it is not recommended to study on cellular physiology and metabolic flux analysis due to the impact of continual environmental changes throughout the fermentation.

Frequently preferred, fed-batch cultivation can be accomplished by the addition of limiting nutrient during the fermentation. A one-stage fed-batch cultivation is adequate for biosynthesis by a growth-associated producer; while a non-growth-associated producer require a fastidious two-stage cultivation. [Yamane *et al.*, 1996b] Conventionally, after being cleanly washed, a well-grown seed culture from the first stage could be inoculated for a better performance of biopolyester synthesis in the second stage.

To obtain relatively high yield and productivity of polyhydroxyalkanoate biosynthesis, it is insisted that the addition of substrate be suitably controlled. [Jang and Rogers, 1996] In practice, strategy for feeding is basically triggered by certain specific changes in the growth medium as a result of depletion of the limiting nutrient. The most customary strategies for feeding are through automatic nutrient-regulated, pH-regulated, DO-stat, and auxostat.

In response to carbon depletion, the level of acidity of the culture medium is somewhat directly changed within certain range. However, it is declared that the pH increase according to glucose depletion is slow for *Ralstonia eutropha* NCIMB 11599. [Madison and Huisman, 1999] Instead, it is suggested that the trigger for further glucose addition be attained through DO-stat strategy. As an illustration, *Table 2.3-2* reveals application of various feeding strategy for fed-batch cultivation for polyhydroxyalkanoate biosyntheses.

Superior to the other modes of operation, continuous cultivation is recommended for a variety of studies on cellular metabolism. Specifically, the application of the continuous culture is preferentially employed for an investigation of physiological aspect [Gostomski and Bungay, 1996] and metabolic flux analysis. [Stephanopoulos *et al.*, 1998] However, due to its high susceptibility of culture contamination and cellular mutation, it is not of such popularity for bioprocess. In addition, difficulty from maintaining the low concentration of certain substances below their inhibition level renders the continuous cultivation unaffectionately employed.

Table 2.3-2 **: Application of various feeding strategy for fed-batch cultivation for polyhydroxyalkanoate biosynthesis.**

Feeding strategy	**Controlled substrate for supplementation**	**Microbial strain**	**Range of application**	**Reference**
Automatic nutrient-regulated	Glucose for P(3HB) biosynthesis	*Ralstonia eutropha*	10-20 g/l	Kim. B.S. *et al.*, 1994
pH-stat	Acetate for P(3HB) biosynthesis	*Ralstonia eutropha* H16	0-1.5 g/l [e]	Shi *et al.*, 1997
pH-stat	Lactate for P(3HB) biosynthesis	*Ralstonia eutropha* H16	0-4.5 g/l [e]	Shi *et al.*, 1997
pH-stat	Acetate for P(3HB) biosynthesis	*Ralstonia eutropha* ATCC 17697^T	Around 1 g/l	Sugimoto *et al.*, 1999
pH-stat	Sucrose and ammonium ion for P(3HB) biosynthesis	*Alcaligenes latus* DSM 1123	10-20 g/l for sucrose and 50-100 mg/l for ammonium ion	Yamane *et al.*, 1996b
pH-auxostat	Glucose and ammonium ion for P(3HB) biosynthesis	*Ralstonia eutropha* ATCC 17697	1-20 g/l for glucose and 1.5 μM-10 mM for ammonium ion	Gostomski and Bungay, 1996

Note : Footnote ‘e’ means the values are not exactly reported but estimated by the author.

2.3.2 Effecting process variable

For several decades, it has been the central issue to develop the most efficient process for biosynthesis of polyhydroxyalkanoate. With a selected microbial producer, effect of such process variables should be thoroughly determined.

Since *Ralstonia eutropha* is a non-growth-associated producer, two groups of process variables can be classified. The first group is responsible for an optimal cell growth; whereas the second is for an optimal synthesis of biopolyester. Certain variables could have a positive influence on cell growth but, undesirably, a negative effect on biopolyester synthesis. Hence, it is urged that conclusion of the effect of any process variables be carefully interpreted.

Generally, the environmental conditions of the fermentation are to be mentioned first; for examples, culture temperature, level of acidity, level of dissolved oxygen, level of dissolved carbondioxide, agitation speed, and aeration rate. Unless specified, the mentioned variables should be reliably kept constant for a desired physiological condition. Listed in Table 2.3-3 are the illustrative process variables for optimal biosynthesis of poly(3-hydroxybutyrate) by two bacterial strains, *Ralstonia eutropha* NCIMB 11599 and *Alcaligenes latus* DSM 1123.

Table 2.3-3 **: Reported values of culture temperature and initial pH value for optimal biosynthesis of poly(3-hydroxybutyrate).**

Process variable	*Ralstonia eutropha* NCIMB 11599	*Alcaligenes latus* DSM 1123
Culture temperature (°C)	34	33
Initial pH	6.7-6.9	6.5

Note : Data for *Ralstonia eutropha* NCIMB 11599 are taken from Nakkharat (1996)
Data for *Alcaligenes latus* DSM 1123 are taken from Grothe *et al.* (1999)

Since an organism is to be cultivated in a reaction medium, it is required that the medium formulation be appropriately defined. One crucial aspect to be carefully considered is the inhibition level of the substance present in the medium. Listed in *Table 2.3-4* are summary of inhibition levels of certain inhibiting substances.

Table 2.3-4 **: Summary of inhibition levels of certain inhibiting substances.**

Microbial strain	**Inhibiting substance**	**Inhibition level**	**Reference**
Ralstonia eutropha ATCC 17697	Acetic acid	1.0 g/l	Sugimoto *et al.*, 1999
Ralstonia eutropha	Propionic acid	0.5 g/l	Kim, J.H. *et al.*, 1992

Various studies were primarily based on the investigation of certain process characteristics; for instances, specific growth rate, specific consumption rate of substrate of interest, specific formation rate of biopolyester, specific formation rate of other products, and any informative yield coefficients. The aim of these studies were seemingly to search for an optimal biosynthesis of polyhydroxyalkanoate by varying the crucial process variables.

Vastly, the variations of the process variables of interest were conducted over a specified range of study. Subsequently, the conclusion was drawn out from a number of data points. Unlike the mentioned procedure, such studies employed a concept of statistics for not only a less-time-consuming experimental design but also a rational strategy for data analysis. Surface response analysis has been introduced for data analysis in a quantitative manner. Review on this statistical tool for the application of bioprocess will be assuredly covered in the final full report if it can be practically used for this research. Instead, the conventional studies for effecting parameters are covered as follows.

Suzuki *et al.* (1986) demonstrated that continuously adding a small amount of ammonia enhanced biosynthesis of poly(3-hydroxybutyrate) by *Pseudomonas* growing on methanol in a fed-batch system.

The effect of controlled concentration of glucose was investigated by Kim, B.S. *et al.* (1994). However, other levels of controlled concentration were not varied and investigated.

A quantitative investigation of level of glucose and ammonium ion was proposed by Ramsay *et al.* (1990). The experimentation was carried out in a chemostat fermentation of *Ralstonia eutrophus*. However, the investigation was based on the variation of feed ratio not directly on the reaction medium. An optimal glucose to ammonium ion feed ratio was 33:1 at a dilution rate of 0.15 per hour. At this ratio, the residual glucose level was 5 gram per liter but the residual ammonium ion concentration was surprisingly not reported.

A complete investigation of level of glucose and ammonium ion in the reaction medium was reported by Gostomski and Bungay (1996). Chemostat fermentation of *Ralstonia eutrophus* ATCC 17697 for poly(3-hydroxybutyrate) biosynthesis was achieved with the dilution rate between 0.07 and 0.24 per hour. Neither glucose nor ammonium ion concentrations had a direct effect on the concentration and yield of biopolyester over a broad ranges of concentrations. They also revealed that glucose concentration above 1 gram per liter affected the growth rate when the culture was nitrogen-limited. This implied the microbe grew according to a non-Monod pattern.

In continuous culture of *Ralstonia eutrophus*, it was reported by Henderson and Jones (1997) that the theoretical maximal yield of poly(3-hydroxybutyrate) on glucose of 0.48 gram per gram can be nearly attained within 5 percents at a growth rate of 0.05 per hour.

Such studied also indicated the importance of the growth rate on the incorporation of 3-hydroxyvalerate unit when a fructose-valerate mixture was used as the substrate in a continuous cultivation. [Koyama and Doi, 1995] At dilution rates varying from 0.06 to 0.32, the content of 3-hydroxyvalerate unit increased from 11-79 percents.

It is informative to compare the studies on polyhydroxyalkanoate biosynthesis by considering the following two terms as rationale criteria, ‘polyhydroxyalkanoate accumulation as the percentage of the cell dry weight’ and ‘monomer composition as the percentage of the polymer’. [Madison and Huisman, 1999] The following studies are reviewed according to the stated criteria.

By varying the ratio of acetate and propionate in the substrate, Doi *et al.* (1987) found that poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) can be accumulated within *Ralstonia eutropha* H16 up to 50 percents of the dry-cell weight. Additionally, the 3HV could be synthesized within the range of 0 to 45 percents.

In contrast, *Ralstonia eutropha* NCIMB 11599 was cultivated in various carbon sources. The 85 percents of 3HV fraction in the copolymer could be observed when valerate was used as a carbon source. Whereas cultivation in mixture of 5-chlorovalerate and valerate yielded the terpolyester containing 3HB, 3HV, and 5HV monomers up to 46 percents of the dry-cell weight and with 52 percents 5HV monomer. [Doi *et al.*, 1987]

2.3.3 Scale of operation

Concerning scale of operation, various studies have been extensively achieved by tons of researchers. Certain studies were conducted as a small laboratory scale to investigate the fermentation characteristics of interest. While some were attempted by a commercial section for mass production of biopolyester in a much more larger scale.

Summarized in *Table 2.3-5* are certain studies of polyhydroxyalkanoate in a laboratory scale. It is to be noted that detailed discussions of the results of some selected studies are already pointed out in the former topic. Consequently, this topic will cover only a pilot and an industrial scale of operation.

First commercialized by W.R. Grace and Co. in the United States of America, poly(3-hydroxybutyrate) was almost exposed to the public through the production and isolation processes developed by Baptist and Werber. [Baptist, 1962a; Baptist, 1962b; Baptist and Werber, 1964; Baptist, 1964; and Baptist and Werber, 1965] Sutures and prosthetic devices were initially innovated. [Baptist and Ziegler, 1965] As well, the unpurified product in the form of plastic laminate was trailed to be commercialized. [Baptist and Werber, 1963] Unfortunately, owing to the fact that the fermentation process yields were relatively low and solvent extraction process was very costly, the project was ultimately abandoned. Moreover, another comment is that the produced polymer was heavily contaminated with bacterial residues; as a result, it was difficult for melting process. Notwithstanding, this project can be realized as the truly pioneering work both in terms of reporting on the plastic potential of poly(3-hydroxybutyrate) and in proposing its use as a biocompatible material. [Baptist and Werber, 1964]

Later on in the nineteen seventies, the energy crisis was worldwidely emerged. It was an incentive to search for naturally-occurring substitutes for synthetic plastics. Imperial Chemical Industries, ltd. (ICI), a renowned British corporation, attempted to commercialize the novel poly(3-hydroxybutyrate). At that time, several bacterial species were evaluated as potential production organisms. The low cost of methanol and ICI's experience with fermentations of methanol utilizers made methylotrophic bacteria the first choice of interest. However, the amount of polymers produced per cell was insufficient and its molecular weight was too low for commercial applications. Then *Azotobacter* was selected for study thanks to its well-known putative production organism. Desperately, the studied strains were unstable and secreted an unacceptably-large amount of by-products in the form of polysaccharide. The third specie of interest was *Ralstonia eutropha*, which was formerly called as *Alcaligenes eutrophus*. Great success of poly(3-hydroxybutyrate) production was observed when culturing on fructose under nitrogen- or phosphate limiting conditions. Up to 70 percents of the dry-cell weight can be synthesized as the high-molecular-weight polymer. The resulting production process yielding the high-molecular-weight biopolymer was in 200,000-litre stirred fermentation vessels. Despite the stated success, commercialization of poly(3-hydroxybutyrate) was impeded by its comparatively high production costs and obscure eminence over conventional petroleum-based plastics. [Howells, 1982]

Copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) was thence in focus by ICI due to its improved properties over the original homopolymer. [Holmes *et al.*, 1982] Subsequently, Biopol ® was the trademark of the copolyester exploited by ICI and Marlborough Technical Management (MTM). [Anonymous, 1987] The copolyester production by a glucose-utilizing mutant of *Ralstonia eutropha* was at a scale of 300 tons a year. Afterwards, the first commercial product made of Biopol ® was launched in Germany as a biodegradable packaging bottle of Wella shampoo. [Anonymous, 1990]

Another pilot operation was started by Chemie Linz GmbH, an Austrian company, poly(3-hydroxybutyrate) production by *Alcaligenes latus*, a growth-associated poly(3-hydroxybutyrate) producer, was carried out from sucrose as a carbon source at up to 1,000 kilograms a week. To improve the properties of the polymer, biologically-safe nucleating agents and plasticizers were used as additives. [Hänggi, 1990; and Hrabak, 1992]

2.4 Metabolic engineering

Superbly, the application of metabolic engineering concept to biosynthesis of polyhydroxyalkanoate was collectively reviewed by Madison and Huisman (1999). However, such information can be assessed as a guiding strategy for development of an engineered organism for efficient biosynthesis of biopolyester. In other words, it focused on the synthesis side of metabolic engineering not the analytical side. While the aim of this research is to accomplish the fundamental of the analytical side of metabolic engineering through the application of metabolic flux analysis.

As for the study of Shi *et al.* (1997), two comments for biosynthesis of poly(3-hydroxybutyrate) by *Ralstonia eutropha* H16 in fed-batch culture was concluded.

First, the metabolic networks for poly(3-hydroxybutyrate) biosynthesis from acetate, butyrate, and lactate as a carbon source were proposed as depicted as *Figure 2.4-1*. It can be observed that these three networks share several similarities; it comprises gluconeogenesis, pentose phosphate pathway, tricarboxylic acid cycle, anaplerotic pathway, oxidative phosphorylation, nitrogen assimilation, biosynthesis of glutamate and glutamine, biosynthesis of PHB, biomass production, and pathway for ATP maintenance. The completely different pathways for substrate uptaking are considered as the only disagreement.

Secondly, the corresponding flux distributions were investigated through two aspects. As for the first aspect, the effect of different phases of cell growth on PHB synthesis was examined, which in turn reflected the effect of nitrogen limitation. It can be noticed that the flux to the tricarboxylic acid cycle through isocitrate dehydrogenase was approximately constant when the cell was culturing in the different stage of fermentation. But an increase flux to the PHB synthesis as well as a decrease flux to glyoxylate bypass can be observed when nitrogen was on-going limited.

Concerning the second aspects, the effect of different carbon sources on PHB synthesis under nitrogen-limiting condition was examined. After normalizing all fluxes in those three flux distributions by butyrate uptake rate, butyrate was likely to be highly suggested for PHB biosynthesis. Since the highest conversion of uptaken substrate to PHB as well as the highest ATP production can be addressed.

Two remarks on this research are as follows. It is somewhat questionable in applicability of stoichiometry representing the biomass production. Due to the lack of relevant information on the microbial cell of interest, the stated stoichiometry is imitated from that of *Escherichia coli*. In addition, the comparison of flux distributions from three carbon sources was susceptibly made regardless of different strategies employed in fermentation. Specifically, constant-feeding strategy was applied to culture on butyrate; while pH-stat strategy was applied to culture on acetate, and the combination of pH-stat and constant-feeding strategy was applied to culture on lactate.

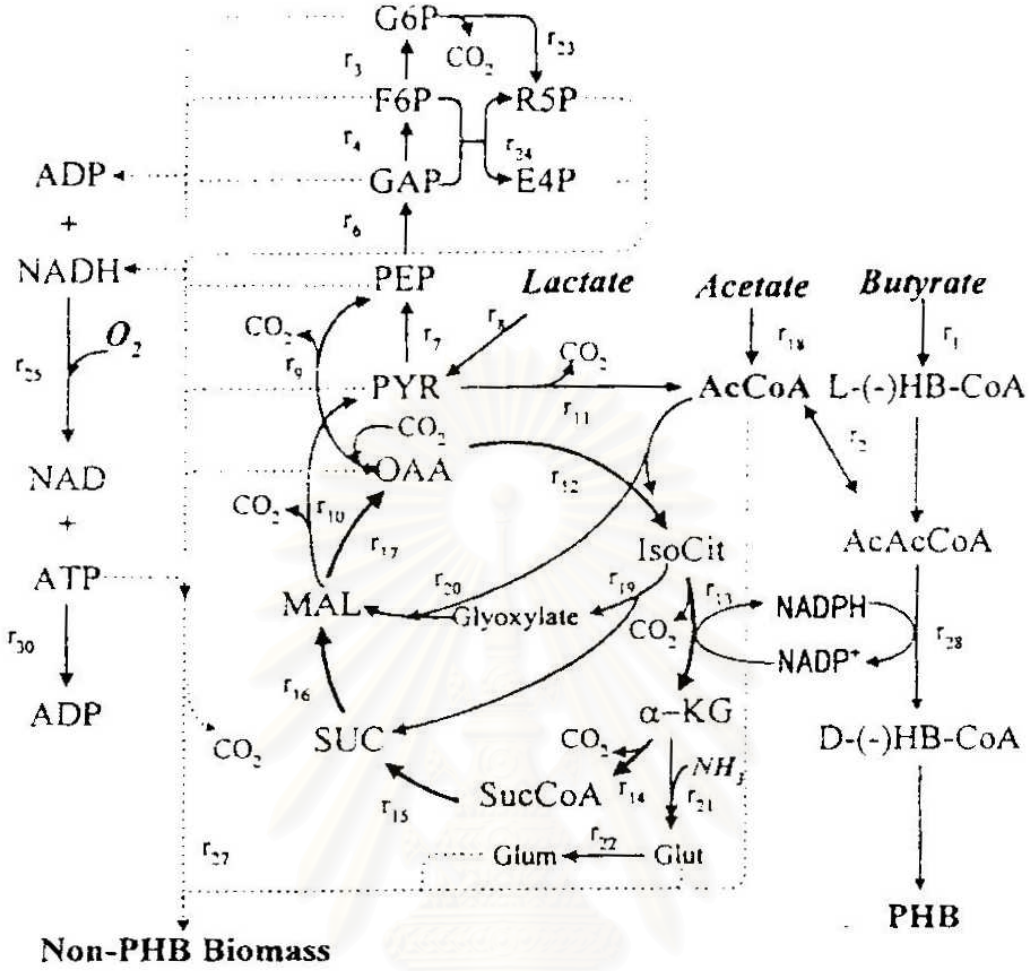


Figure 2.4-1 **: Proposed schematic diagram of poly(3-hydroxybutyrate) biosynthesis from acetate, butyrate, and lactate. [Shi, 1997]**

Another application of metabolic flux analysis to poly(3-hydroxybutyrate) biosynthesis was reported by Wong *et al.*. (1999) Recombinant *Escherichia coli* transformed with the PHB production plasmid pSYL107 was cultivated on the glucose-based minimal medium in the high-cell-density fed-batch fermentation. The final dry-cell weight was 227 grams per liter with a final PHB content of 78 percent. Despite the slow growth rate, the overall productivity was 3.39 grams of PHB per liter of broth per hour.

Then the effect of restricted oxygen supply was investigated through the application of metabolic flux analysis. Although excretion of by-product acetate and lactate could be observed during the severe oxygen limitation, the specific PHB formation rate was not significantly impaired.

Concerning the pyruvate fermentation by *Torulopsis glabrata* IFO 0005, biphasic fed-batch fermentation was developed as the efficient production of pyruvate through the application of metabolic flux analysis. [Hua *et al.*, 2001]

Specifically, at the first stage, initial glucose concentration should be relatively low to enhance the cell growth. Because high level of glucose concentration at the first stage could lead to glucose repression, which can be realized as an undesired physiological state for cell growth. While the dissolved-oxygen level should be relatively high so that the tricarboxylic acid cycle, which is directly linked with the biomass formation, would be highly active. In

addition, thiamine concentration should also be suitably high to enhance the activity of pyruvate dehydrogenase complex, the key enzyme dealing with the catabolism of pyruvate. The stated suitably high amount of thiamine should be carefully denoted since it cannot be physically decreased during the fermentation. Its overwhelmed amount could undesirably increase the activity of the pyruvate dehydrogenase complex in the second stage of fermentation which is not of anticipation.

Then during the second stage of pyruvate production, it is desired to reduce the flux through both pyruvate dehydrogenase complex and pyruvate decarboxylase while still maintaining the glycolytic flux in the high level. As a result, glucose concentration can be controlled at the high level. This could intentionally keep the glycolytic flux fairly high, although the higher flux through pyruvate decarboxylase is to be sacrificed. Additionally, low level of dissolved oxygen was recommended during the second stage owing to its contribution to reduction of the flux through the pyruvate dehydrogenase. Unfortunately, excretion of ethanol, another undesired by-product, could result from the decreasing content of oxygen. This could be simply solved by the addition of nicotinic acid which may be capable of relaxing the requirement for NADH oxidation by the alcohol dehydrogenase pathway.

It is noteworthy that the stated pyruvate production may be somewhat useful for this research. Because Lee *et al.* (1994) reported that as propionate concentration in reaction medium increased, an enhancement in pyruvate excretion was observed along with a decrease in the yield of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis.

In conclusion, the aim of most researches on polyhydroxyalkanoate is the development of the most efficient production process in the most economically feasible way. This requires the application of the concept of both modern biotechnology and sophisticated genetic engineering.

Starting by the selection of the most promising organism, the succeeding stages can be foregoing accomplished. First of all, it is to be noticed that different polyhydroxyalkanoate producers are capable of producing different types of the biopolymer. As formerly mentioned, biopolymer type has a major influence on the property characteristics. Consequently, the first attentive issue is which producer is to be selected for the study to produce the specified type of biopolymer whose desired property characteristics.

Although naturally-occurring polyhydroxyalkanoate producers are eminent for high biopolymer yielding prospects, they are comparatively incompetent in high biopolymer productivity prospects due to their relatively low tolerance for substrate inhibition.

On the other hand, recombinant producers are renowned for their fast-growing capability. As a result, higher cell productivity can be markedly attained. However, the major pitfalls of the production by recombinant producers are their lower biopolymer yielding aspects and the unpredictable side effects from the expression of the cloned genes.

In the meanwhile, the biopolymer production by transgenic plant has been considered as the most sustainable production processes since there is no such biological waste generated in the process. Nonetheless, substantially low yield is the obstacle for the production by this technology. Thus it is still the long way to make the transgenic process attractively feasible.

The next issue to be addressed is which substrate is to be fed into the process. Certain producers are capable of producing either homopolymer or copolymer in response to the uptaken substrates. Instead of cofeeding of more than one substrate, such copolymers can be completed by feeding only sugar for certain versatile producers. Specifically, these versatile producers possess notable genes responsible for synthesizing certain key precursor metabolites essential in copolymer production by themselves without any necessities

obtaining from metabolizing other cosubstrates which might cause inhibitory effect on cells. Cloning of those genes has also been remarkably focused by several researchers.

As well, there have been a great number of researches on biopolymer production from other valueless substrates like agricultural and industrial wastes. Unsurprisingly, the unattractive productions can be perceived due to the toxicity of those impured substrates. However, this approach is interesting as the alternative for ideal waste management in the future.

Chemolitrotrophic cultivations by certain chemolitrotrophs have also been extensively studied. Relatively low biopolymer production is still the major bottleneck due to the application of the highly-risky explosive gas mixture which is urged to be operated under the substantially low level for safety concern. The understanding of this type of cultivation may someday be useful for the somewhat resemble cultivation, novel transgenic production.

Another issue of interest involved with polyhydroxyalkanoate production is the development of the production process itself. Improvement of the biopolymer yield and productivity can be achieved through the development of novel feeding strategies. In additiuon, various fermentation conditions have also been of popularity. Specifically, medium formulation has been extensively investigated.

At first, various nutrient limitations have been employed to investigate certain physiological stages of the cells. From these, quantitative determination of the present substrates for the sake of medium formulation will be further elucidated. Frequently speaking, the initial concentrations of the substrates are of interest. As time elapsed, the profiles of the parameters can be analyzed.

There is still another approach involving the medium formulation. It is demanded that the concentrations of the key substrates be kept constant to exactly ensure the same physiological stage of the cells. A more logical explanation of the celluar activities can be drawn out from this approach. Unfortunately, it requires much more sophisticated and reliable process control in the experimentation.

Interestingly, all stated issues could be elucidated with the application of the newly-emerging concept of metabolic engineering. Saying, the analytical side of metabolic engineering can be fully in service for achieving those purposes. While the synthesis side deals with the genetic manipulation to obtain the specified polyhydroxyalkanoate producers.

Extensively, the application of metabolic engineering to variety of bioprocesses can be observed. For either selected environmental or genetic manipulations, rational suggestions can be concluded for obtaining a more promising production process. For instances, it could recommend which and to what extent the substrate should be used in the process. Which and to what extent certain compounds should be added to enhance the productivity. Which gene expression should be either amplified or repressed to enhance the productivity.

Unfortunately, the application to polyhydroxyalkanoate production has not been vastly achieved. However, the following suggestion is somewhat interesting. From the genetic point of view, it was reported that the biopolymer biosynthesis might be regulated by the three sequential encoded genes; namely, 3-ketothiolase, acetoacetyl coenzyme A reductase, and PHA synthase. Simple maximization of the activity of all three is not the solution for maximization of the biopolymer production. The first is maximized by increasing the rate of glycolysis; however, an increase in glycolytic flux will reduce the flux into the pentose phosphate pathway and, hence, NADPH regeneration. Therefore, maximization of polyhydroxyalkanoate production could be achieved by optimally balancing the flux distribution at the glucose-6-phosphate branch point rather than straightforward amplification of the three enzymes. [Stephanopoulos *et al.*, 1998]

Table 2.3-5 **: Certain studies of polyhydroxyalkanoate in a laboratory scale.**

Microbial strain	**Cultivation technique**	**Substrate**	**Cultivation time (hr)**	**Cell concentration (g/l)**	**Biopolymer concentration (g/l)**	**Biopolymer content (%)**	**Copolymer content (%)**	**Cell productivity (g cell/l/hr)**	**Biopolymer productivity (g biopolymer/l/hr)**	**Reference**
A.latus DSM 1123	PH-stat fed-batch	Sucrose	18	143	71.4	50	0	7.94	3.97	Yamane *et al.*, 1996b
R.eutropha	PH-stat fed-batch	Glucose	30	122	79.3	65	0	4.07	2.64	Lee, Y.W. and Yoo, Y.J., 1994
R.eutropha ATCC 17697	Fed-batch with microfiltration unit	Fructose	64	88.01	42.10	47.83	0	N/A	0.66	Apichart, 1996
R.eutropha ATCC 17697	Fed-batch	Fructose	50	60.82	10.9	17.92	0	N/A	0.22	Vingvon, 1996
R.eutropha ATCC 17697^{T}	Recycled-gas closed-circuit batch	$H_2/O_2/CO_2$	40	91.3	61.9	68		2.28	1.55	Tanaka *et al.*, 1995
R.eutropha ATCC 17697^{T}	- Heterotrophic cultivation by pH-stat high-cell-density fed-batch - Autotrophic cultivation by Recycled-gas closed-circuit batch	- Acetic acid - $H_2/O_2/CO_2$	- 21 - 83	- 48.6 - 22.9	- N/A - 12.6	- N/A - N/A	- 0 - 0	- N/A - N/A	- N/A - N/A	Sugimoto *et al.*, 1999.

***Table 2.3-5* (cont.) : Certain studies of polyhydroxyalkanoate in a laboratory scale.**

Microbial strain	Cultivation technique	Substrate	Cultivation time (hr)	Cell concentration (g/l)	Biopolymer concentration (g/l)	Biopolymer content (%)	Copolymer content (%)	Cell productivity (g cell/l/hr)	Biopolymer productivity (g biopolymer/l/hr)	Reference
R.eutropha NCIMB 11599	Glucose-control fed-batch	Glucose	50	164.0	121.0	76		3.28	2.42	Kim, B.S. *et al.*, 1994.
R.eutropha NCIMB 11599	Fed-batch	Glucose and valeric acid	50	N/A	90.4	N/A	3HV 20.4	N/A	1.80	Lee, I.Y. *et al.*, 1995.
R.eutropha NCIMB 11599	Fed-batch	Glucose	74	281	232	82	0	N/A	3.14	Ryu *et al.*, 1997
Recombinant *E.coli*	PH-stat fed-batch	Glucose and LB	42	117.0	89.0	76	0	2.79	2.11	Kim, B.S. *et al.*, 1992
Recombinant *E.coli*	Fed-batch	Glucose and oleic acid	74	116	70.2	60.5	0	N/A	0.95	Lee, S.Y. *et al.*, 1995

CHAPTER 3
THEORETICAL BACKGROUND

3.1 Cellular metabolism
3.2 Fermentation
3.3 Metabolic engineering

The prospect of relevant theoretical background can be categorized into three main sections; namely, cellular metabolism, fermentation and metabolic engineering.

As for the first section, basic knowledge and idea on cellular metabolism will be briefly summarized. Crucially, cellular metabolism can be recognized as fundamental background of the other two sections. While fermentation section is indeed involved with handling of massive primary experimental data. Computation of those primary data is of great importance in acquisition of reliable and informative data for the succeeding section. The last section, metabolic engineering, can be inevitably considered as the powerful and useful tool for informational extraction of experimental data. Specifically, the latter section deals with the analysis of the corresponding experimental data directly obtained from the former section, fermentation approach.

Consequently, rational discussion and conclusion which is the main aim of this research can be made as concomitant of all stated three parts; cellular metabolism, fermentation and metabolic engineering approach.

3.1 Cellular metabolism

As for cellular metabolism approach, there are two different frameworks to be discussed; biochemistry framework and metabolic engineering framework.

Based on the knowledge of biochemistry, much more delicate studies on cellular metabolism have been intensively explained. The biochemistry framework is, theoretically, applied to elucidate how the living cell functions or undergoes any cellular activities; such as, cell growth and product formation. Specifically, it emphasizes on the causes of the cellular functioning. Accordingly, it can be used as inceptive reference for the sake of the latter, metabolic engineering framework.

On the other hand, metabolic engineering framework focuses on any consequences of cellular metabolism. According to the specified perturbation on cellular metabolism, the obtained result is whether as expected or not. In addition, simplification of the relevant idea and model can be addressed.

It is worthwhile informing that the following issues will cover only metabolism of *Ralstonia eutropha* and related strains leaving metabolism of other living cells beyond this scope of work.

3.1.1 Biochemistry framework

A living cell comprises a large number of different compounds and metabolites. Clearly, those compounds and metabolites can be classified into two main constituents; namely, water and water-free constituent. Usually known as dry cell weight biomass, the content of water-free cellular constituents are presented in the form of macromolecular pools of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), protein, lipid, and carbohydrate. As tributes from several independent reactions, synthesis and organization of the stated macromolecular pools

into a functioning cell can be successfully accomplished. [adapted from Stephanopoulos *et al*, 1998]

Principally, those independent reactions can be categorized into two contributions; namely, catabolism and anabolism. *Figure 3.1-1* reveals schematic diagram of general connection of catabolism and anabolism. It can be noticed that the central metabolites connecting catabolism and anabolism are twelve precursor metabolites and currency metabolites. Listed in *Table 3.1-1* are all stated central metabolites and their contributions in cellular metabolism. In addition, illustrative cellular metabolism from sugar is depicted as *Figure 3.1-2*.

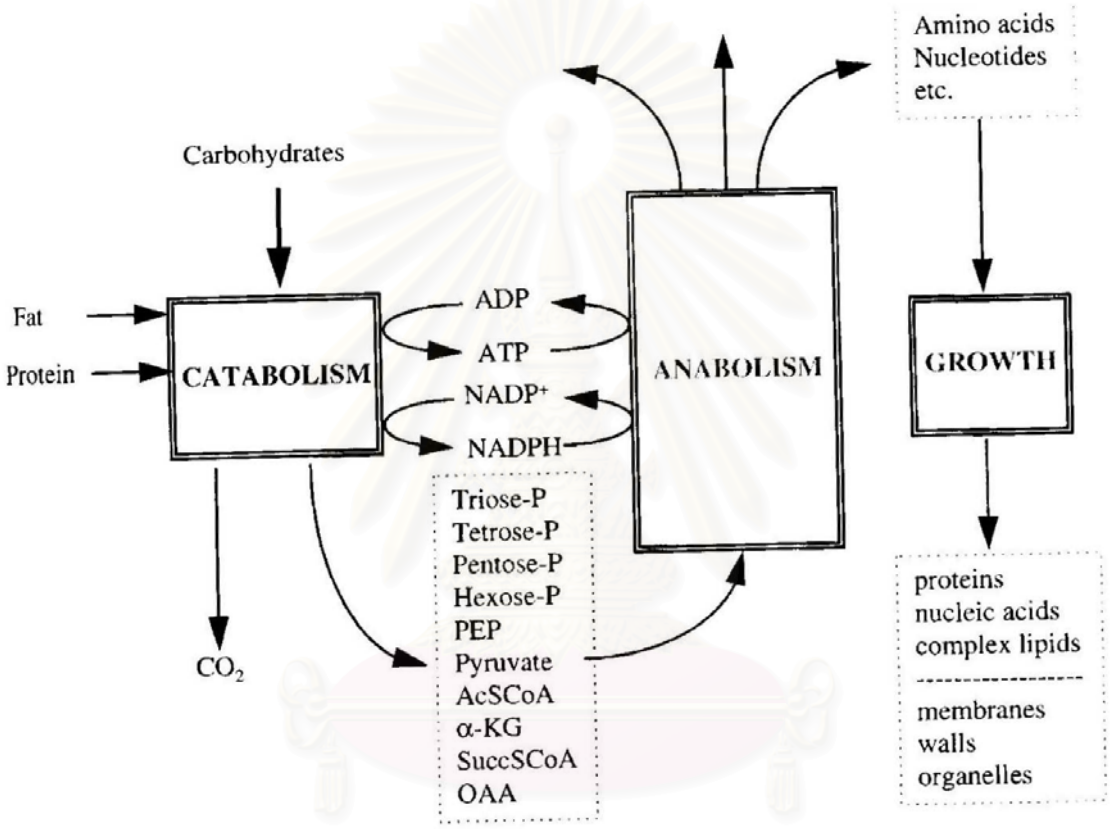


Figure 3.1-1 **: Schematic diagram of general connection of catabolism and anabolism.** [Stephanopoulos *et al*, 1998]

Table 3.1-1 **: List of all precursor metabolites and currency metabolites and their contributions in cellular metabolism.**

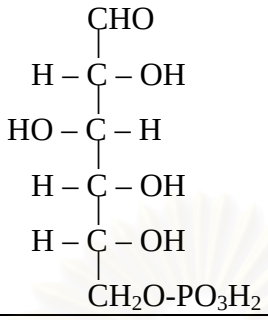
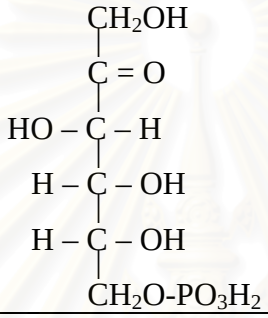
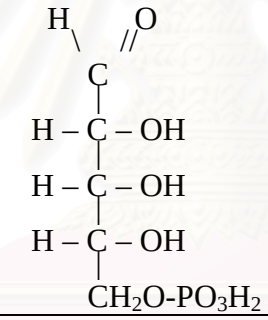
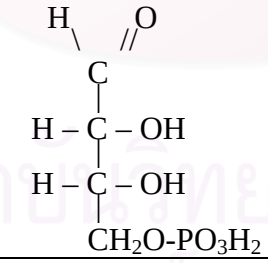
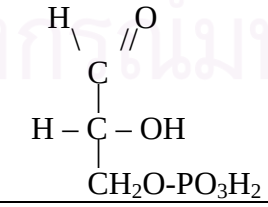
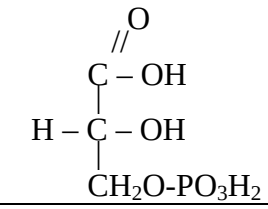
Central metabolite	**Chemical structure**	**Major biosynthetic function**
Glucose-6-phosphate		Central intermediate in many catabolic pathways; carbohydrate polymers
Fructose-6-phosphate		N-acetylglucosamine, N-acetylmuramic acid murein
Ribose-5-phosphate		Nucleic acid, histidine
Erythrose-4-phosphate		Aromatic amino acids
3-phosphoglyceraldehyde		Precursor to dihydroxyacetone; lipids
3-phosphoglyceric acid		Beginning point for the serine family of amino acids

Table 3.1-1 **(cont.) : List of all precursor metabolites and currency metabolites and their contributions in cellular metabolism.**

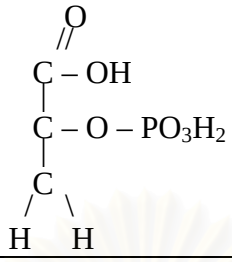
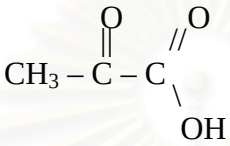
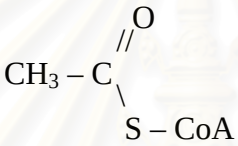
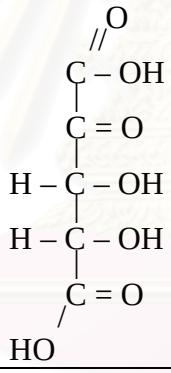
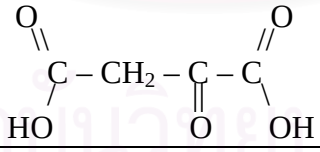
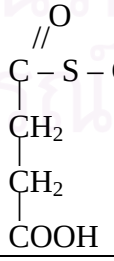
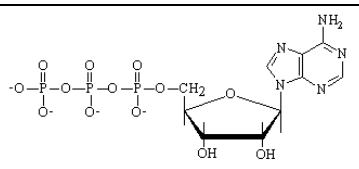
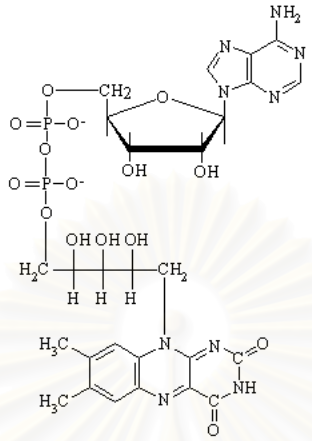
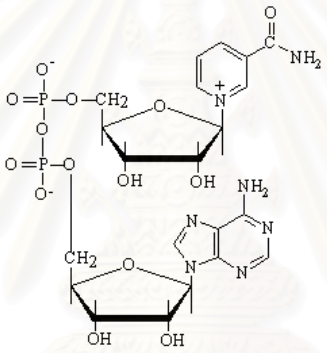
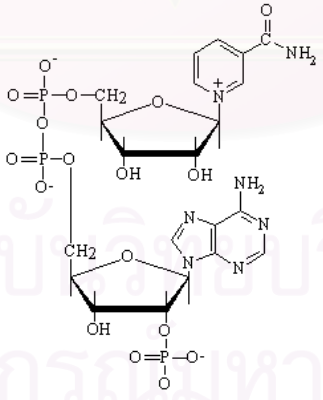
Central metabolite	Chemical structure	Major biosynthetic function
Phosphoenol pyruvic acid		Key intermediate in several carbohydrate degradation sequences; aromatic amino acids precursor
Pyruvic acid		Key intermediate in degradative metabolism; "gateway compound" to the TCA cycle; precursor to the pyruvate family of amino acids
Acetyl coenzyme A		Key energy compound; beginning point for lipid synthesis; participant in many synthetic processes
α-ketoglutarate		TCA cycle intermediate; precursor to the glutamate family of amino acids
Oxaloacetate		TCA cycle intermediate; precursor to the aspartic family of amino acids
Succinyl coenzyme A precursor to heme		Key intermediate in the TCA cycle; major energy intermediate
Adenosine triphosphate		Energy equivalent

Table 3.1-1 **(cont.) : List of all precursor metabolites and currency metabolites and their contributions in cellular metabolism.**

Central metabolite	Chemical structure	Major biosynthetic function
Flavin adenine dinucleotide		Strong currency metabolite in oxidative energy-yielding-metabolism
Nicotinamide adenine dinucleotide		Currency metabolite in energy yielding metabolism presenting in oxidative respiration
Nicotinamide adenine dinucleotide phosphate		Reducing equivalent in anabolic biosynthesis

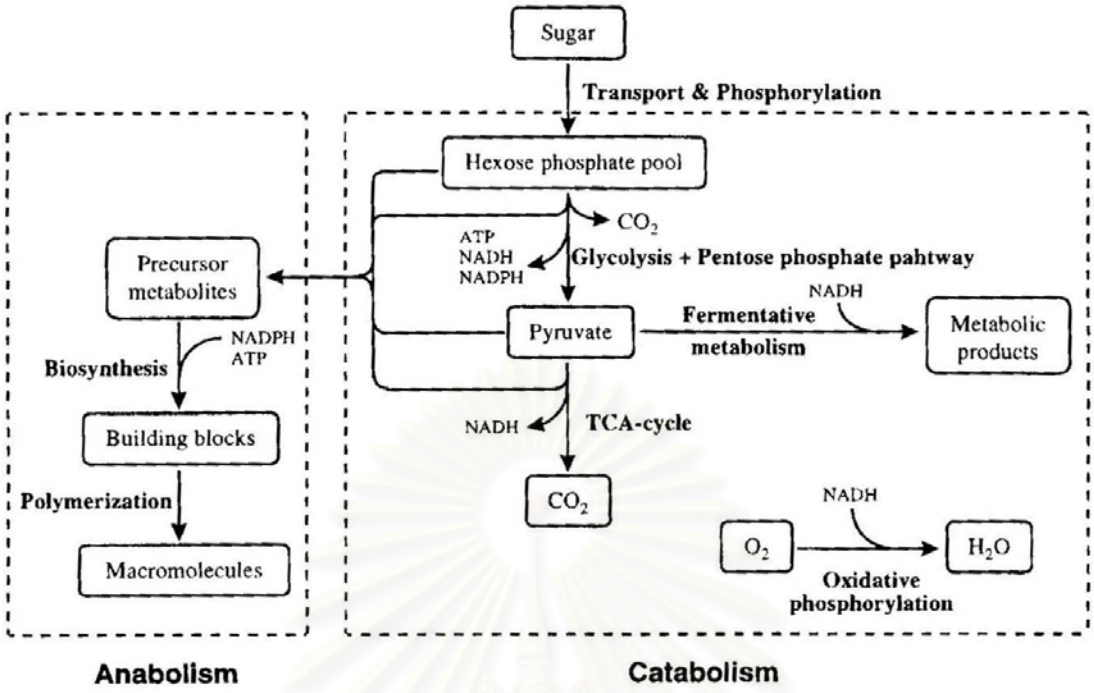


Figure 3.1-2 **: Illustrative cellular metabolism from sugar.** [Stephanopoulos *et al*, 1998]

Also referred to as fueling reactions, catabolism can be defined as cellular mechanism involves with degrading and oxidizing substrates. All catabolic pathways are responsible for production of twelve key precursor metabolites needed in biosynthesis, Gibbs free energy in terms of adenosine triphosphate (ATP) used in all cellular activities, and reducing power in the form of nicotinamide adenine dinucleotide (NADPH) necessary in biosynthesis.

Consecutively, anabolism can be realized as cellular mechanism dealing with formation of all involved macromolecules. To accomplish cellular steps of biosynthesis and polymerization, all anabolic pathways begin with one of twelve key precursor metabolites accompanied by the utilization of free energy and reducing power in either direct or indirect manner.

Concise discussion of all involved mechanism for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from glucose and/or propionic acid as the carbon source and ammonium sulfate as the nitrogen source will be covered as follows.

3.1.1.1 Catabolism

General prospects

To accomplish three stated purposes; provision of precursor metabolites, free energy, and reducing power, cellular catabolism is to be carried out. As for ordinary bacteria, catabolism is started as soon as the carbon and energy sources are taken up through the substrate-uptaking process.

Also referred to as a central metabolic pathway, catabolism of carbon and energy sources can be categorized into five groups; namely, catabolism of carbohydrates, catabolism of other compounds, fermentative pathway, tricarboxylic acid cycle (TCA cycle), anaplerotic pathway, and respiratory pathway. From the given carbon and energy sources of interest, the initial starting point of the representative pathways, which are best describing the most probable metabolic network, can be addressed. Glucose and gluconate; for examples, are likely to be catabolized for the first time through a carbohydrate catabolism. And then they are further dealt with either a TCA cycle or a fermentative pathway with the supplementation of an anaplerotic pathway. While propionic acid, the well-known fatty acid, is subject to a catabolism of organic acid first and then accompanied by a reversed-carbohydrate catabolism, a TCA cycle, and an anaplerotic pathway. A demonstrative scheme representing the entrance of selected carbon and energy sources are illustrated as *Figure 3.1-3*.

As a general citation, illustrated as *Figure 3.1-4* is the overall relationship between the major carbohydrate pathways and the tricarboxylic acid cycle coupling with electron transport through either respiratory pathway or fermentative pathway.

Physiological role

Collectively, the discussion on the corresponding physiological role of the key catabolic pathways as well as the associative key precursor metabolites and currency metabolites are concisely covered as in *Table 3.1-2*.

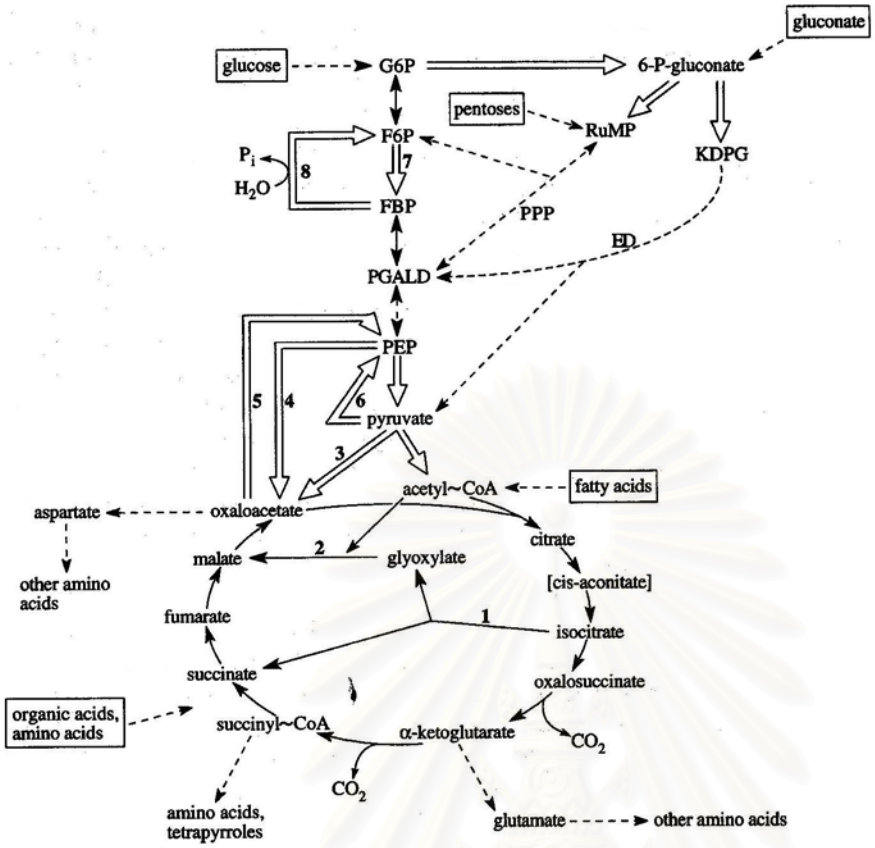


Figure 3.1-3 **: Demonstrative scheme representing the entrance of selected carbon and energy sources.** Enzymes : (1) isocitrate lyase; (2) malate synthase; (3) pyruvate carboxylase; (4) PEP carboxylase; (5) PEP carboxykinase; (6) PEP synthetase or pyruvate phosphodikinase; (7) phosphofructokinase; (8) fructose-1,6-bisphosphatase. [White, 2000]

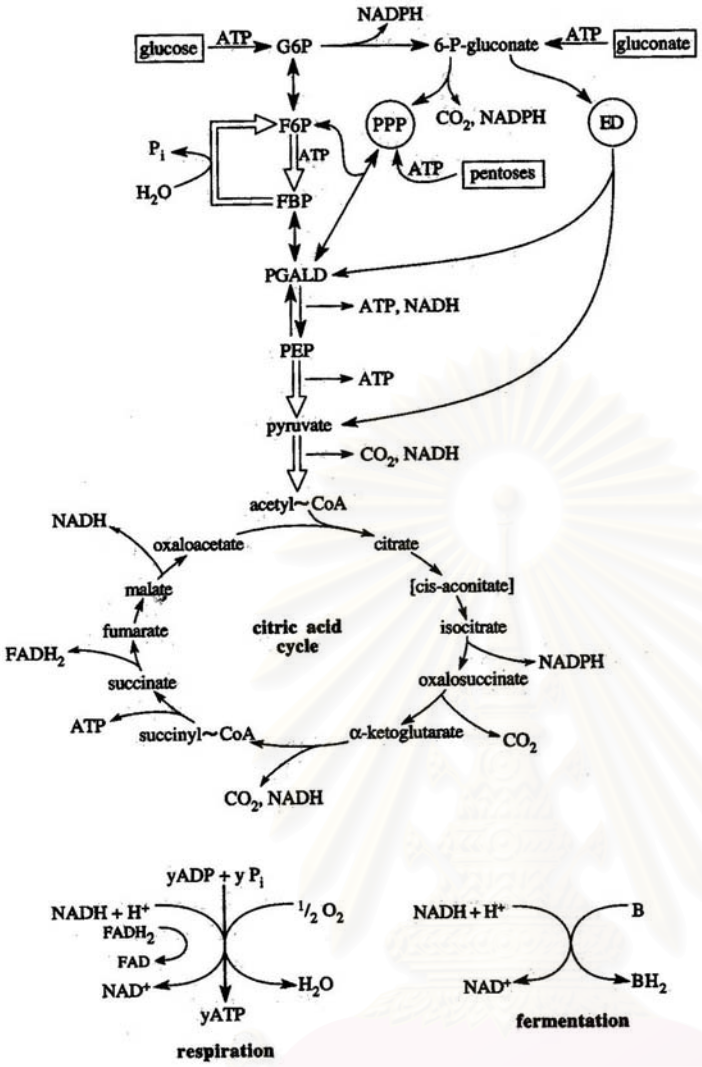


Figure 3.1-4 **: Overall relationship between the major carbohydrate pathways and the tricarboxylic acid cycle coupling with electron transport through either respiratory pathway or fermentative pathway.** [White, 2000]

Table 3.1-2 **: Concise review of the catabolic pathways.**

Metabolic pathway	**Physiological role**	**Associative key precursor metabolites and currency metabolites**
Embden-Meyerhof-Parnas (EMP) pathway	Catabolism of glucose via fructose-6-phosphate yielding two moles of pyruvate, two moles of NADH, and a net two moles of ATP.	- Glucose-6-phosphate - Fructose-6-phosphate - 3-phosphoglyceraldehyde - 3-phosphoglycerate - Phosphoenolpyruvate - Pyruvate - ATP / ADP pairs - NADH / NAD pairs
Pentose-phosphate (PP) pathway	Catabolism of glucose via glucose-6-phosphate, from each glucose-6-phosphate catabolized, yielding one mole of 3-phosphoglyceraldehyde, three moles of carbondioxide, and six moles of NADPH.	- Ribose-5-phosphate - Erythrose-4-phosphate - 3-phosphoglyceraldehyde - NADPH / NADP pairs
Entner-Doudoroff (ED) pathway	Catabolism of glucose via glucose-6-phosphate yielding two moles of pyruvate, one mole of ATP, one mole of NADPH, and one mole of NADH.	- Glucose-6-phosphate - 3-phosphoglyceraldehyde - 3-phosphoglycerate - Phosphoenolpyruvate - Pyruvate - ATP / ADP pairs - NADH / NAD pairs - NADPH / NADP pairs
Methylcitric acid (MCA) pathway	Catabolism of propionic acid yielding one mole of pyruvate and one mole of succinate, which is reoxidized to oxaloacetate by the TCA cycle.	- Pyruvate

Table 3.1-2 **(cont.) : Concise review of the catabolic pathways.**

Metabolic pathway	**Physiological role**	**Associative key precursor metabolites and currency metabolites**
Gluconeogenesis	Reversed glycolytic pathway to fructose-6-phosphate from either pyruvate or phosphoenolpyruvate.	- Fructose-6-phosphate - 3-phosphoglyceraldehyde - 3-phosphoglycerate - Phosphoenolpyruvate - Pyruvate - ATP / ADP pairs - NADH / NAD pairs
Tricarboxylic acid (TCA) or Krebb's cycle	Oxidative decarboxylation of pyruvate yielding one mole of acetyl coenzyme A, one mole of carbondioxide, and one mole of NADH followed by the successive stage of cyclic oxidation of acetyl coenzyme A yielding another two moles of carbondioxide, one mole of ATP, two moles of NADH, one mole of NADPH, and one mole of $FADH_2$.	- Acetyl coenzyme A - Oxaloacetate - α-ketoglutarate - Succinyl coenzyme A. - ATP / ADP pairs - NADH / NAD pairs - NADPH / NADP pairs - $FADH_2$ / FAD pairs
Fermentative pathway	Energy-incapable reoxidation of NADH via pyruvate, which is active in most anaerobic cells and certain aerobic cells culturing on some limiting conditions.	- NADH / NAD pairs

Table 3.1-2 **(cont.) : Concise review of the catabolic pathways.**

Metabolic pathway	**Physiological role**	**Associative key precursor metabolites and currency metabolites**
Anaplerotic pathway	➢ Carboxylation of pyruvate to form oxaloacetate active in certain aerobic cells growing on sugar.	➢ Replenishment of oxaloacetate.
	➢ Carboxylation of phosphoenolpyruvate to form oxaloacetate active in certain aerobic cells growing on sugar or other carbon sources incapable of directly providing acetyl coenzyme A.	➢ Replenishment of oxaloacetate.
	➢ Oxidation of malate to form pyruvate.	➢ Replenishment of oxaloacetate and drainage of carbon out of the TCA cycle.
	➢ Glyoxylate cycle as the condensation of two molecules of acetyl coenzyme A to form succicate, involved with metabolism of acetate and fatty acids where acetyl coenzyme A is a common intermediate.	➢ Bypassing the oxidation of α-ketoglutarate.
Respiratory pathway	Energy production and reoxidization of NADH and $FADH_2$ via the terminal electron acceptor yielding certain moles of ATP according to the number of active coupling site.	- ATP / ADP pairs - NADH / NAD pairs - $FADH_2$ / FAD pairs

Pathway diagram

In addition, diagrammatic representations of certain selected pathways are illustrated as follows. It is noteworthy that the selected pathways are tentative to be involved in the catabolism of glucose and propionic acid by *Ralstonia eutropha*, the bacterial strain of interest.

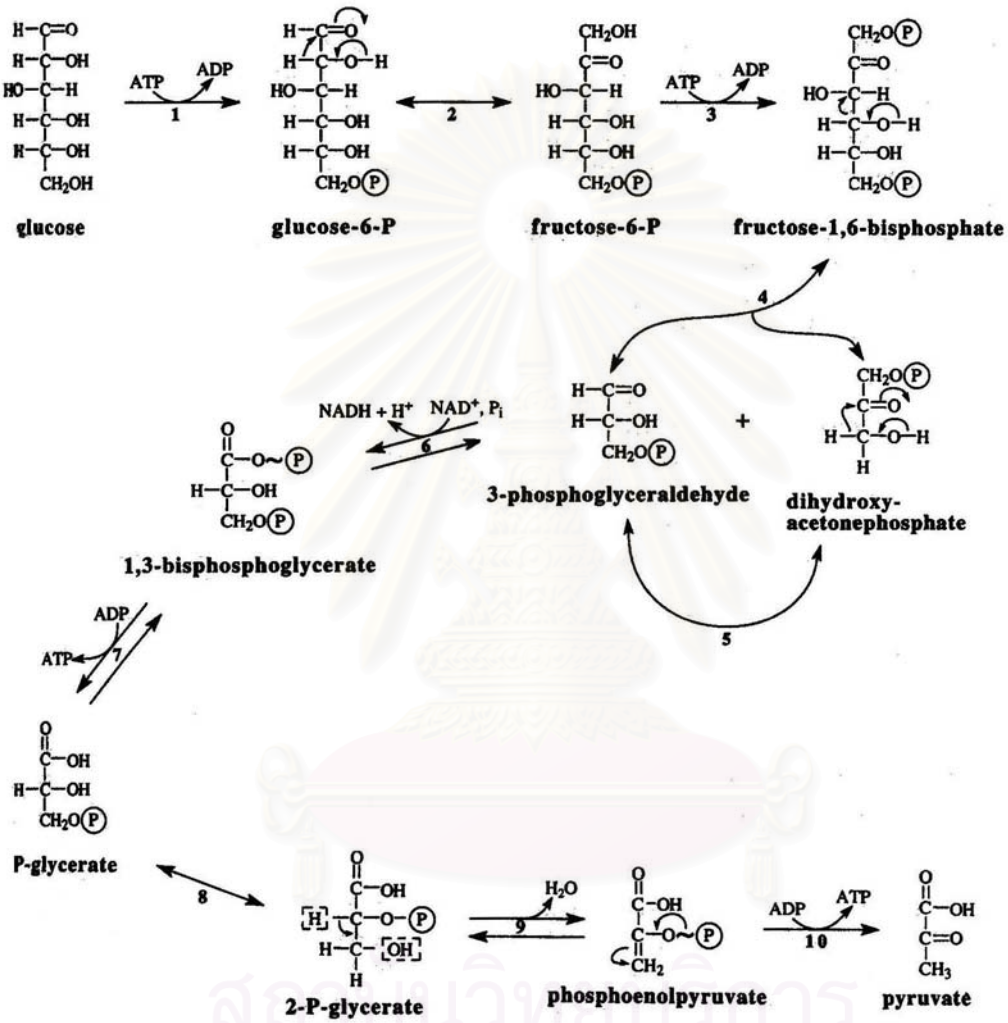


Figure 3.1-5 **: Schematic diagram of Embden-Meyerhof-Parnas (EMP) pathway and the associative precursor metabolites.** Enzymes: (1) hexokinase; (2) isomerase; (3) phosphofructokinase; (4) fructose-1,6-bisphosphate aldolase; (5) triosephosphate isomerase; (6) triosephosphate dehydrogenase; (7) phosphoglycerate kinase; (8) mutase; (9) enolase; (10) pyruvate kinase. [White, 2000]

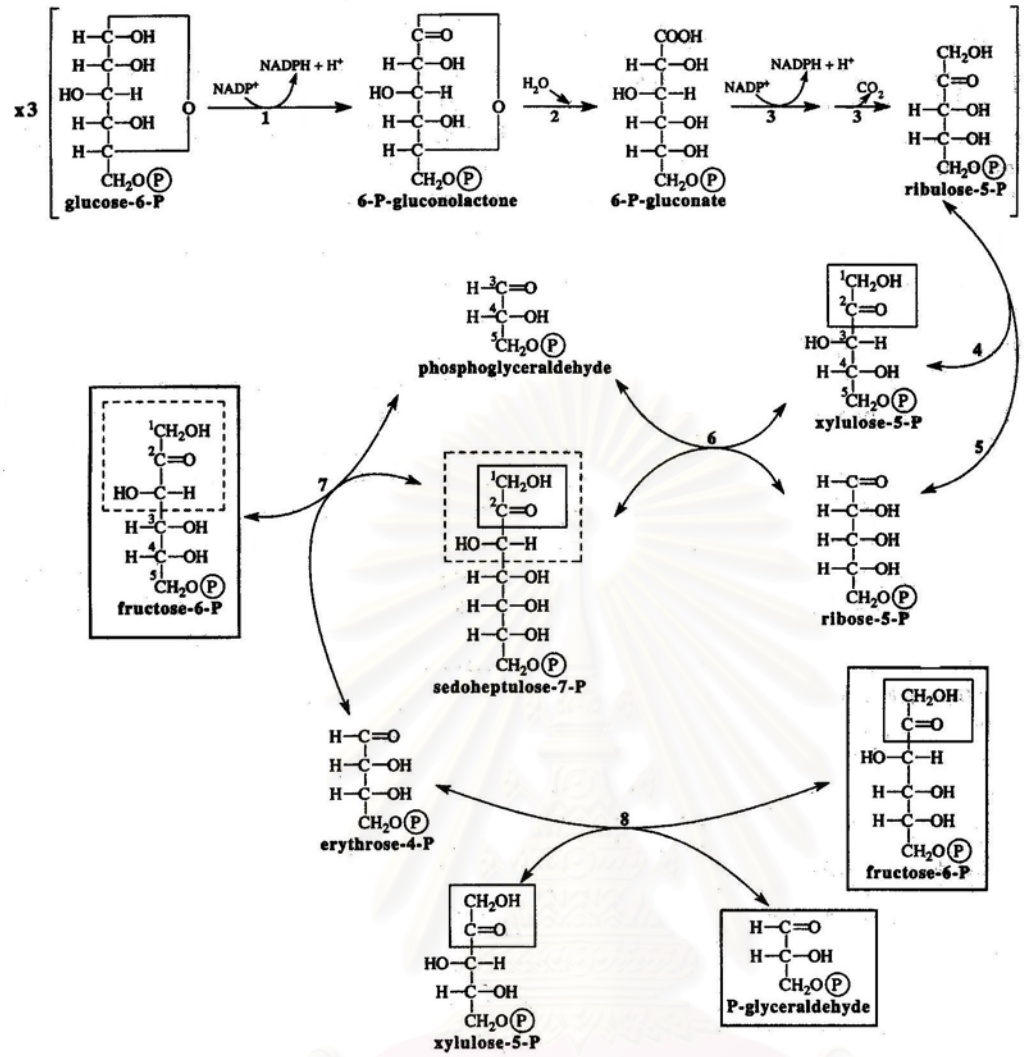


Figure 3.1-6 **: Schematic diagram of pentose-phosphate (PP) pathway and the associative precursor metabolites.** Enzymes: (1) glucose-6-phosphate dehydrogenase; (2) lactonase; (3) 6-phosphogluconate dehydrogenase; (4) ribulose-5-phosphate epimerase; (5) ribose-5-phosphate isomerase; (6) transketolase; (7) transaldolase; (8) transketolase [White, 2000]

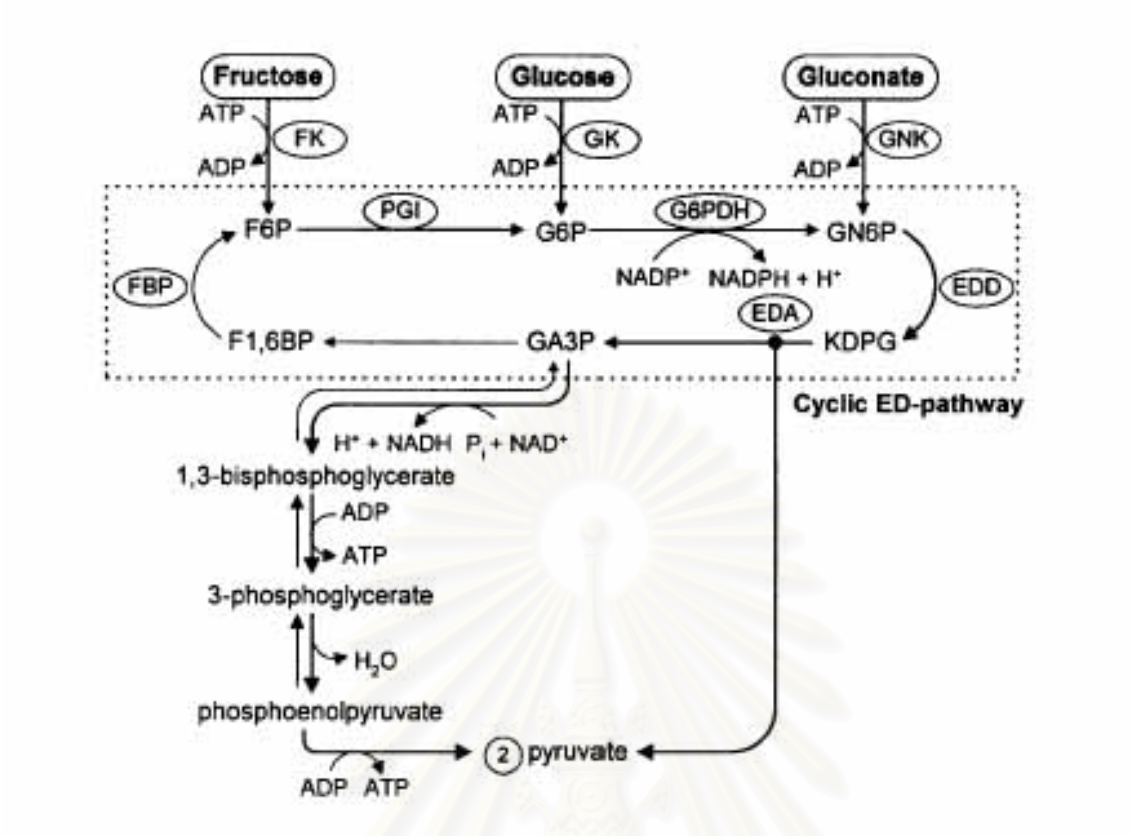


Figure 3.1-7 **: Schematic diagram of cyclic mode of Entner-Doudoroff (ED) pathway and the associative precursor metabolites.** Intracellular metabolites: F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GN6P, gluconate-6-phosphate (or 6-phosphogluconate); KDPG, 2-keto-3-deoxy-6-phosphogluconate; GA3P, glyceraldehyde-3-phosphate; F1,6BP, fructose-1,6-bisphosphate. Enzymes: FK, fructokinase; GK, glucokinase; GNK, gluconokinase; PGI, phosphoglucoisomerase; G6PDH, glucose-6-phosphate dehydrogenase; EDD, 6-phosphogluconate dehydratase (or ED-dehydratase); EDA, KDPG aldolase (or ED-aldolase); FBP, fructose-1,6-bisphosphatase. All other enzymes outside the box are exactly the same as those in the EMP pathway. [adapted from Joo, 1998]

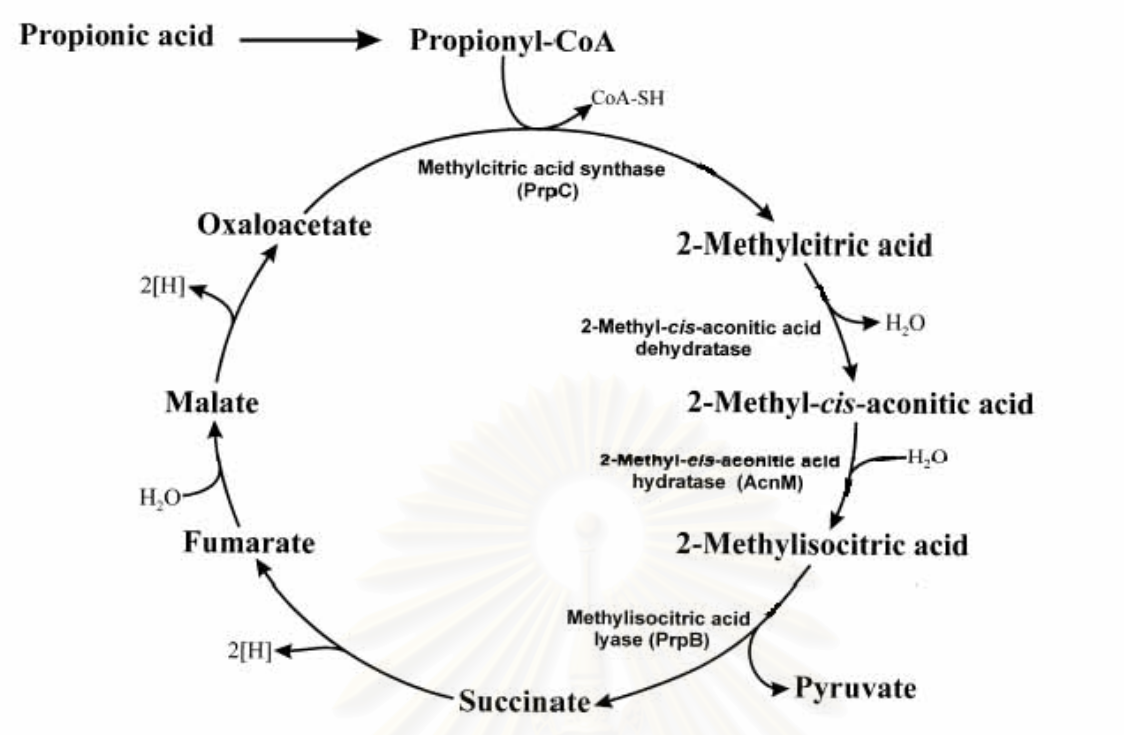


Figure 3.1-8 **: Schematic diagram of methylcitric acid (MCA) cycle.** [adapted from Br™mer and Steinb⟩chel, 2001]

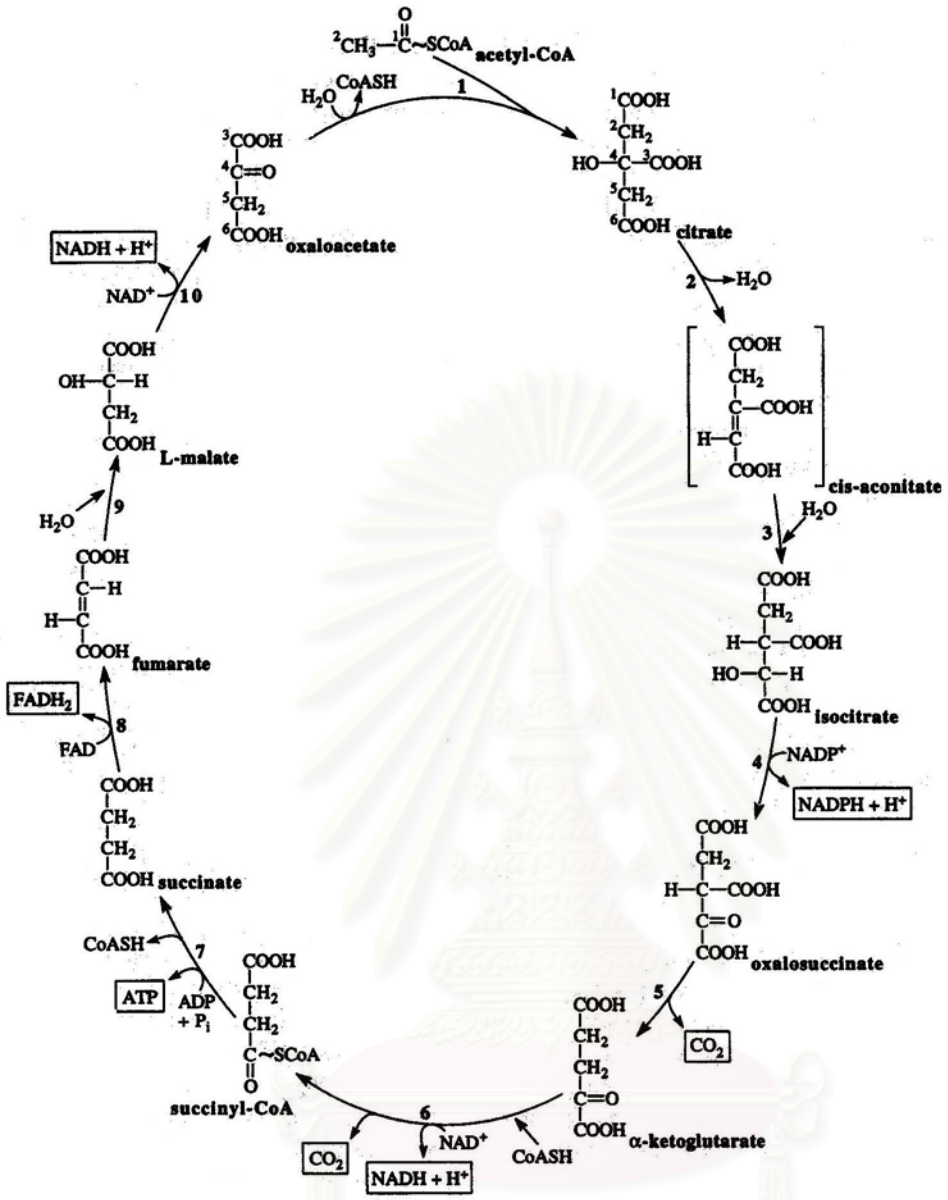


Figure 3.1-9 **: Schematic diagram of oxidative tricarboxylic acid (TCA) cycle and the associative precursor metabolites.** Enzymes: (1) citrate synthase; (2) aconitase; (3) aconitase; (4) isocitrate dehydrogenase; (5) isocitrate dehydrogenase; (6) α-ketoglutarate dehydrogenase; (7) succinate thiokinase; (8) succinate dehydrogenase; (9) fumarase; (10) malate dehydrogenase. [White, 2000]

3.1.1.2 Anabolism

As for a general description, anabolism is responsible for cellular synthesis. To achieve this responsibility, two hierarchical steps are to be successfully conceded; namely, biosynthesis of crucial building blocks and polymerization of those building blocks.

Due to completely different reaction steps occurring in different types of microbial cells, merely broad explanations will be mentioned throughout the concept of biosynthesis and polymerization of crucial building blocks.

Preferentially, based on the information of well-known microbial cells, general expressions for describing cellular anabolism can be drawn out to universally apply to other microbial cells. For instances, for microbial cell of interest, if there is no specific information on both biosynthesis and polymerization of protein, DNA, and RNA, the suggestive expressions for those of *Escherichia coli* can be pictorially picked up as representative idea.

Nevertheless, a more gigantic picture of cellular anabolism is frequently of interest. Lumping all steps of biosynthesis and polymerization of crucial building blocks renders the overall reaction of interest representing a formation of cellular constituents. This simplification is worldwide applicable to most researches on biochemical processes where no details of biochemistry is not of such importance. Also, researches on metabolic engineering sometimes exploit this simplification.

For polyhydroxyalkanoate-biosynthesizing bacteria, two portions are practically treated as cellular constituents; polyhydroxyalkanoate and the rest as a residual biomass. Consequently, general citation of biosynthesis and polymerization steps will be covered merely for those two cellular constituents; those for residual biomass and those for storage component as polyhydroxyalkanoate, respectively.

❑ Biosynthesis and polymerization of residual biomass constituents

Acquiring twelve key precursor metabolites and global currency metabolites from cellular catabolism, biosynthesis of crucial building blocks can be further proceeded. Among those crucial building blocks, which are about seventy-five to one hundred groups, the following are typically mentioned; amino acids, nucleic acids, fatty acids, building blocks of carbohydrate, and building blocks of other storage compounds. [Ingraham *et al*, 1983] These building blocks will be further undergoing polymerization step to essentially form the residual biomass constituents. Concise discussion will be covered as follows.

Routinely, amino acids can be recognized as the building blocks of proteins as well as precursors for other biosyntheses. The starting point of the biosynthesis of amino acids focuses on how nitrogenous compound can be incorporated into the microbial cell.

Although there are more than twenty kinds of amino acids to be synthesized by the cells, a few key amino acids can be directly synthesized by ammonia assimilation [Moat and Foster, 1988] Specifically, certain α-keto acids like α-ketoglutarate, in the presence of the appropriate enzyme, can be converted to α-amino acids like L-glutamate by the addition of ammonia. And after L-glutamate biosynthesis, biosynthesis of other amino acids can be successively accomplished.

Precedently, there are two approaches fulfilling the biosynthesis of L-glutamate from the presence of ammonia and α-ketoglutarate; the one catalyzed by a NADP-linked glutamate dehydrogenase (GDH) and the other catalyzed by the combination of glutamine synthase (GS) and glutamate synthase, respectively (previously known as glutamine amide-2-oxoglutarate aminotransferase : GOGAT). Schematic diagram of both are illustrated in *Figure 3.1-10*.

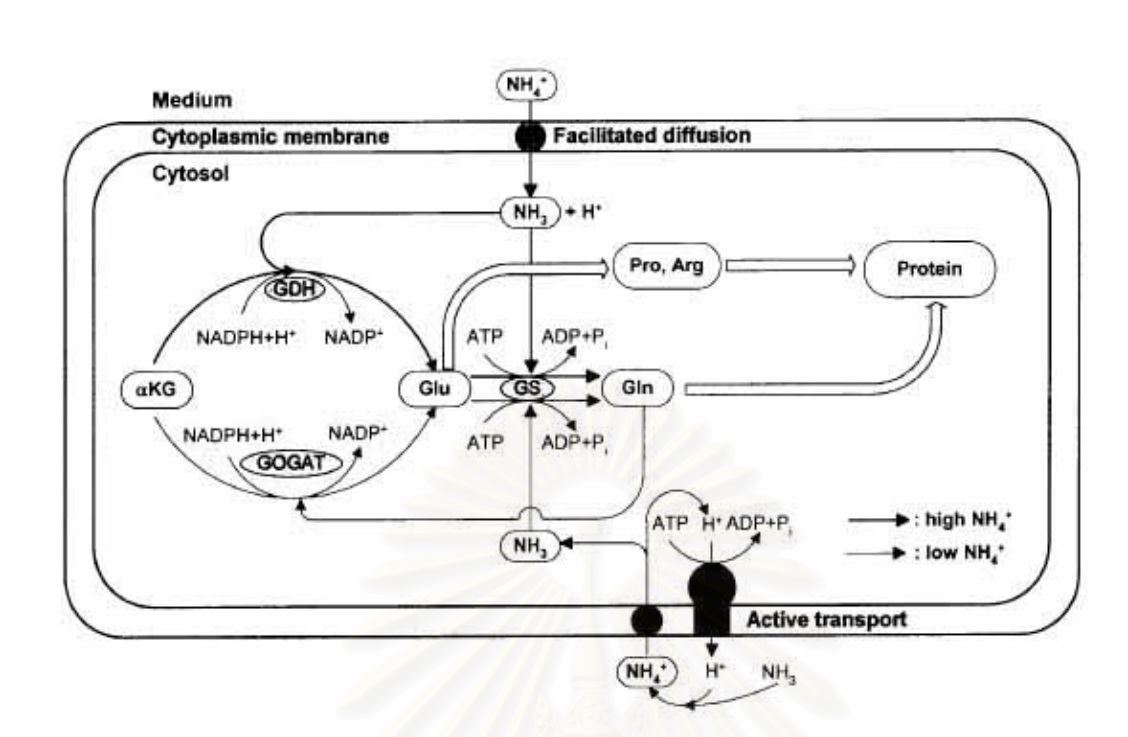


Figure 3.1-10 **: Schematic diagram of ammonium assimilation pathway.** Intracellular metabolites: αKG, α-ketoglutarate; Glu, glutamate; Gln, glutamine; Pro, proline; Arg, arginine. Enzymes: GDH, NADPH-linked glutamate dehydrogenase; GOGAT, NADPH-linked glutamate synthase; GS, glutamine synthetase. [adapted from Joo, 1998]

Interestingly, both systems are capable of L-glutamate biosynthesis from ammonia and α-ketoglutarate with the oxidation of NADPH. But as for the second system, the GS-GOGAT system, energy in the form of ATP is needed to be hydrolyzed for each L-glutamate formed. As a result, the GS-GOGAT system is a high-affinity system for ammonia assimilation.

In addition, the GS-GOGAT system is mainly active at low ammonia concentration because glutamate synthase is repressed by ammonia. As well, it is involved with the regulation of biosynthesis of several nitrogen-containing compounds because L-glutamine always serves as a nitrogen donor. Thus, L-glutamine can be realized as an important branch point in various cellular metabolism. On the other hand, the GDH system is active at comparatively high ammonia concentration and is not involved with the regulation of biosynthesis of nitrogen-containing compounds.

As formerly stated, biosynthesis of the other amino acids and the other biomass constituents followed by the corresponding polymerization step can be successively accomplished after two key amino acids are synthesized. Detailed discussion of the biosynthesis of those is not shown hereby. Instead, the overall formation of residual biomass constituents will be responsible for those omitted reaction steps.

Practically, the stoichiometry of the overall formation of residual biomass constituents can be obtained by a deliberate study of cellular components, saying elemental analysis of the cell. With the information from the elemental analysis, anabolic stoichiometry can be obtained as a contribution of the key precursors and key amino acids that matches the biomass formula. Adapted from the information of the well-known *Escherichia coli*, the following expression, proposed by Shi *et al* (1997), is the overall formation of residual

constituents that matches the biomass formula of *Ralstonia eutropha* as $C_4H_{6.9}N_{0.98}O_{1.63}$ with 5-percent ash, which was reported by Ampe *et al* (1996).

$$\text{Biomass} + 4.11\text{ADP} + 0.751\alpha\text{-KG} + 0.826\text{NADP} + 0.312\text{NADH} + 0.261\text{CO}_2 - 0.021\text{G6P} - 0.007\text{F6P} - 0.09\text{R5P} - 0.036\text{E4P} - 0.013\text{GAP} - 0.15\text{G3P} - 0.052\text{PEP} - 0.283\text{PYR} - 0.374\text{AcCoA} - 0.179\text{OAA} - 0.832\text{GLUT} - 0.025\text{GLUM} - 4.11\text{ATP} - 0.826\text{NADPH} - 0.312\text{NAD} = 0 \quad \textbf{--- (3.1.1)}$$

❑ Biosynthesis and polymerization of polyhydroxyalkanoate

Regardless of amino acids, nucleic acids, fatty acids, and carbohydrate building blocks, there are still varieties of building blocks to be synthesized by microbial cells. Usually, those building blocks may be synthesized as optional storage. Specifically, carbon and energy storage is frequently regarded in certain microbial cells.

Polyhydroxyalkanoate is of such popularity as carbon and energy storage accumulated as a macromolecular granule inside certain living cells. Merely, metabolic pathway for biosynthesizing polyhydroxyalkanoate in the form of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) is already covered as *Figure 2.2-2*.

3.1.2 Metabolic engineering framework

Four key words are to be mentioned in metabolic engineering framework; time frame of interest, stoichiometry, reaction rate, and simplification of metabolic network.

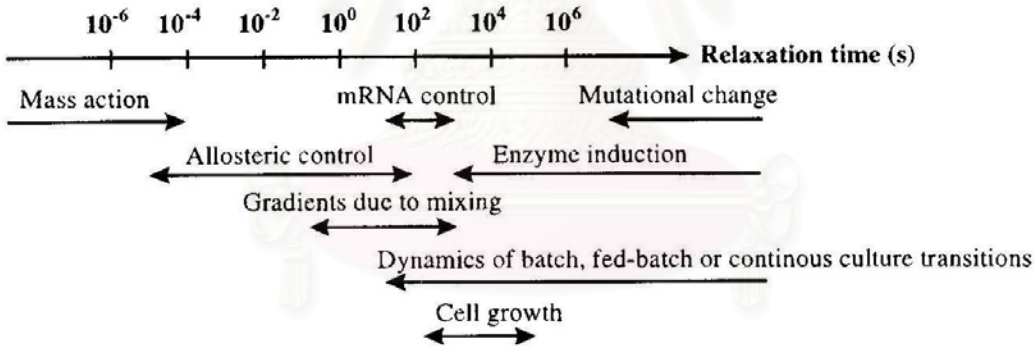
3.1.2.1 Time frame of interest

Theoretically, various dynamic behaviors of cellular metabolism can be extensively perceived. Among those dynamics, merely reactions and pathways of comparable time scale may be studied further in metabolic engineering leaving others which are of totally different time scale out of consideration. [Stephanopoulos *et al*, 1998]

Defined as the characteristic time of the reaction approximated as a first-order process, relaxation time is the central key for specifying the time frame of interest. Practically, the relaxation time of the cell growth is underlying as the reference time frame of interest. Typical relaxation times of various cellular processes are summarized as in *Figure 3.1-14*.

Any reactions and pathways operating much faster than the time frame of interest, specifically operating at one-third of the relaxation time of interest, can be applied with the pseudo-steady state hypothesis. This implies the much faster reactions are rapidly approaching to their equilibrium. Certain enzyme-catalyzing reactions are subject to this category.

On the other hand, any reactions and pathways executing much slower than the time frame of interest, in order of ten times the specified relaxation time, can be neglected due to



its insignificantly small impact on cellular growth. Typically, mutation is of this category; thus, it is not considered in performing any procedures of metabolic engineering.

Figure 3.1-11 **: Typical relaxation times of various cellular processes.** [Stephanopoulos *et al*, 1998]

3.1.2.2 Stoichiometry

As for a general formalism, all substances appearing in cellular metabolism can be mainly classified into four categories; namely, substrate, metabolic product, biomass constituent, and intracellular metabolite. [Stephanopoulos *et al*, 1998]

A substrate can be considered as a compound present in the sterile medium that can be further metabolized by the cell. Preferentially, the stated substrate can be confined to only three main groups; a group providing carbon source, nitrogen source, and energy source. Generally, glucose can be realized as the substrate providing carbon and energy sources; while ammonia is supplied as the source of nitrogen.

Defined as a metabolic product, a compound is successfully synthesized by the cell and significantly excreted into the surrounding medium. Metabolic product can be realized as primary metabolic product, secondary metabolic product, or even heterologous protein which is secreted during cell growth.

Straightforwardly, biomass constituent is recognized as any coordination that makes up biomass. Various macromolecular pools can be perceived as biomass constituents; for instances, RNA, DNA, protein, lipid, and carbohydrate as genuine cellular components as well as biopolymer, polysaccharide, and non-secreted heterologous protein as macromolecular products accumulated inside the cell.

As for the last, intracellular metabolite includes all other compounds merely found inside the cell. Recalled as pathway intermediates, all intracellular metabolites presenting in the living cell can rapidly undergo complete conversion comparing with the on-going change of biomass constituents. Consequently, the pseudo-steady state assumption can be interestingly applicable to any conversion of all intracellular metabolites.

From those stated groups, compounds of interest in the given biochemical reactions can be classified. As a generalization, a typical biochemical comprises N sorts of substrates, M sorts of metabolic product, Q sorts of macromolecular pools making up as biomass constituents, and K sorts of intracellular metabolites. Like ordinary chemical reactions, all biochemical reactions are subject to the principle of stoichiometry. With the application of stoichiometric concept; subsequently, the general formalism of the corresponding compounds involving with the j^{th} reaction with respect to their groups of interest is as follows.

$$\sum_{i=1}^{N} \alpha_{ji} S_i + \sum_{i=1}^{M} \beta_{ji} P_i + \sum_{i=1}^{Q} \gamma_{ji} X_{macro,i} + \sum_{i=1}^{K} g_{ji} X_{met,i} = 0 \qquad \text{---- (3.1.2)}$$

Where α_{ji}, β_{ji}, γ_{ji}, and g_{ji} is the corresponding stoichiometric coefficient of the i^{th} substrate, the i^{th} metabolic product, the i^{th} macromolecular biomass constituent, and the i^{th} intracellular metabolite, respectively.

Considering all J reactions, the following expression in the matrix form can be attained.

$$\overline{\overline{A}}\,\underline{S} + \overline{\overline{B}}\,\underline{P} + \overline{\overline{\Gamma}}\,\underline{X}_{macro} + \overline{\overline{G}}\,\underline{X}_{met} = 0 \qquad \text{---- (3.1.3)}$$

Hereby, $\overline{\overline{A}}$, $\overline{\overline{B}}$, $\overline{\overline{\Gamma}}$, and $\overline{\overline{G}}$ is the so-called stoichiometric matrix of substrates, metabolic products, macromolecular biomass constituents, and intracellular metabolites, respectively. Additionally, the corresponding substrates, metabolic products, macromolecular biomass constituents, and intracellular metabolites are gathered in the form of one-column vector.

3.1.2.3 Reaction rate

Next, the concept of metabolic flux is to be stated. Focusing on the rate of the corresponding compounds of interest resulting from the j^{th} reaction will yield the following expressions.

$$r_{s,i} = -\sum_{j=1}^{J} \alpha_{ji} v_j \quad \text{---- (3.1.4)}$$

$$r_{p,i} = \sum_{j=1}^{J} \beta_{ji} v_j \quad \text{---- (3.1.5)}$$

$$r_{macro,i} = \sum_{j=1}^{J} \gamma_{ji} v_j \quad \text{---- (3.1.6)}$$

$$r_{met,i} = \sum_{j=1}^{J} g_{ji} v_j \quad \text{---- (3.1.7)}$$

Where $r_{s,i}$, $r_{p,i}$, $r_{macro,i}$, and $r_{met,i}$ refers to the net conversion rate, with respect to the j^{th} reaction, of the i^{th} substrate, i^{th} metabolic product, i^{th} macromolecular biomass constituent, and i^{th} intracellular metabolite, respectively. Preferentially applying either dry-cell weight or residual dry-cell weight as a reference, those net conversion rates can be recalled as specific uptake rate of substrate, specific formation rate of metabolic product, specific net formation rate of macromolecular biomass constituent, and specific net formation of intracellular metabolite, respectively. For this study, all specific rates are present based on the residual dry-cell weight. In addition, the stated rates imply the relative amounts into which the corresponding compound is converted.

Likewise, accounting into all *J* reactions, the following expressions in matrix notation can be successfully collected.

$$\bar{r}_s = -\bar{\bar{A}}^t \bar{v} \quad \text{---- (3.1.8)}$$

$$\bar{r}_p = \bar{\bar{B}}^t \bar{v} \quad \text{---- (3.1.9)}$$

$$\bar{r}_{macro} = \bar{\bar{\Gamma}}^t \bar{v} \quad \text{---- (3.1.10)}$$

$$\bar{r}_{met} = \bar{\bar{G}}^t \bar{v} \quad \text{---- (3.1.11)}$$

It is worthwhile realizing that each component of the overall conversion rate vector, $\bar{v}$, can be nominated as the so-called pathway flux or metabolic flux, which will be frequently mentioned.

3.1.2.4 Simplification of metabolic network

Obviously, metabolic network for describing the biological system of interest can be accomplished by integration of all relevant biochemical reactions through the concept of biochemistry. Frequently, the network becomes a set of complexity and cannot provide all informative idea easily as expected.

Amazingly, in metabolic engineering framework, all unimportant complications can be eliminated out of the model of interest. This can be done by simply lumping the straight-chain series of biochemical reactions altogether. The lumped equation still represents those reactions without loosing any information.

Consequently, the network not only turns to be much more simple but also reveals the important interconnection of branch pathways. This can be done providing that the pseudo-steady state hypothesis is not violated. This hypothesis will be revisited in the next topic of this chapter, metabolic engineering approach.

3.2 Fermentation

As for fermentation approach, there are two main constitutional aspects to be discussed; namely, narrative-based aspect and mathematical-modeling-based aspect. Detailed discussion of both aspects will be extensively covered as follows.

3.2.1 Narrative-based aspect

A fed-batch culture can be simplified as a batch culture which is supplied with fresh nutrient. The stated fresh nutrient can be regarded as any growth-limiting substrates or any additives like precursors to products. Occasionally, the terms 'extended culture' and 'semi-batch culture' have also been used to describe this culture technique.

Neither a constant-volume batch culture nor a continuous culture is suitable for production of a non-growth associated product. High concentration of both cell and non-growth associated product cannot be achieved in a single stage of operation. Instead, a two-staged operation is to be employed for an efficient production. As the first stage, it is to build up a high concentration of the cell in a batch mode. And then the metabolism of the cell is rationally switched so that the desired product could be preponderantly promoted in the succeeding stage. This switch may be accomplished by changing any environmental conditions of the culture; for instance, nutrient limitation, so that the cell growth is halted. Noteworthy, it is required that the culture be fed with a just sufficient amount of carbon and oxygen for cellular maintenance and product biosynthesis.

Recently, fed-batch culture technique has been used for the production of antibiotics, vitamins, amino acids, enzymes and growth hormones. As well, application of fed-batch culture to biodegradable polymers has been extensively employed.

Listed in *Table 3.2-1* are the prominences and constraints of the typical fed-batch culture.

Table 3.2-1 **: The prominences and constraints of the typical fed-batch culture.**

Prominences	Constraints
- It is advisable to be applied if substrate inhibition is imposed on the culturing. - It occasionally overcomes problems associated with catabolic repression. - It offers the increasing productivity of biomass, growth-associated products, and non-growth-associated products. - It is recommended to be applied to any cultures with high-viscosity - Contamination, mutation, and plasmid instability can be scarcely found comparing with continuous culture	- Substantial skills of operator are inevitably needed. - Additional instruments for feedback control may be costly. - In case of absence of proper feedback control, the growth pattern of the organism may be significantly deviated and may not always follow the growth profile as expected.

Particularly, process variables are to be monitored during the time course of fermentation. Once they are measured, frequently, they are used as the key parameters for process controlling. According to the control parameters, the techniques for process control can be classified into two main groups; those with feedback control and those without feedback control.

As for the techniques with feedback control, there are two methods fulfilling this purpose; indirect feedback control and direct feedback control. Indirect feedback control is involved with monitoring any fermentation parameters which are directly related to the substrate which may or may not be limiting; for instances, dissolved oxygen, respiratory quotients, and pH value. While direct feedback control can be realized as direct monitoring of the concentration of substrate in the culture broth. Interestingly, the substrate can be either kept at a constant value or can be varied to maintain an optimum concentration.

Concerning the fermentation system without feedback control, certain information like the predetermined feed rate of the specified substrate is to be available so that the appearing growth pattern is kept in track. Practically, the stated feed rate can be either added at a constant rate, at an exponential rate accordance with the increasing biomass formation, or simply intermittent as an incremental addition.

3.2.2 Mathematical-modeling-based aspect

In general, the purpose of fermentation modeling is to bring order to the mass of data which results from a practical fermentation experiment and to express the results in a concise form which is intelligible to colleagues and to those who wish to make use of the results. One of the principle uses of a fermentation model is to design large-scale fermentation processes using data obtained from small-scale fermentations.

3.2.2.1 Material balance equations

The material balance equation is an accounting relationship between the well-defined rates with which a quantity of interest is involved. Typical balance equation can be obtained as follows.

Rate of accumulation = rate in - rate out

Rate in = bulk flow into the volume
+ generation within the volume
+ transfer into the volume across the boundaries other than by bulk flow

Rate out = bulk flow out of the volume
+ consumption within the volume
+ transfer out of the volume across the boundaries other than by bulk flow

Once applying the stated balance equation with set of relevant substances, the following typical balance equations can be obtained as summarized in *Table 3.2-2*.

Table 3.2-2 **: General material balances of typical bioreactor system.**

Viable cells (no cells entering)

$$\frac{d(VX_v)}{dt} = 0 + VX_v r_x - F_{out} X_v$$

Substrate

$$\frac{d(VC_s)}{dt} = F_{in} C_{s,in} - VX_v r_S - F_{out} C_s$$

Product

$$\frac{d(VC_p)}{dt} = F_{in} C_{p,in} + VX_v r_p - F_{out} C_p$$

3.2.2.2 Stated assumptions

It is usual to start off by making a variety of simplifying assumptions. The stated assumptions are as follows.

- The laboratory fermenter is well-mixed; as a result, conditions are uniform throughout the whole of its interior;
- Culturing cells do not die or become non-viable;
- The only two substrates of importance are the carbon source and nitrogen source;
- Oxygen is provided in excess of the need for metabolism and energy production.
- All specific rates are constant in the period of interest.

3.2.2.3 Fed-batch fermenter model

Regarding fed-batch operation for a production of non-growth-associated product, the mathematical model of the fed-batch fermenter based on the material balance concept with the stated assumptions can be summarily covered as follows.

***Table 3.2-3* : General material balances of typical fed-batch bioreactor system.**

Cell balance

$$\frac{d(VX_v)}{dt} = VX_v r_x$$

Substrate balance

$$\frac{d(VC_s)}{dt} = -VX_v r_S + F_{in} C_{s,in}$$

Product balance

$$\frac{d(VC_p)}{dt} = VX_v r_p$$

Volume balance

$$\frac{dV}{dt} = F$$

It can be noticed that the above material balance expressions are in the form of differential equation. To obtain the specific rates, these expressions are to be transformed into an algebraic equation system. Hereby, in this thesis we transform these differential equations by an integral method. As such, these transformed expressions as shown in *Table 3.2-4* are ready for evaluation of the corresponding specific rates by a conventional linear least square method. Essentially, it can be seen that the expressions are somewhat the same like those used for evaluation of typical batch bioreactor system. This is because an intermittently-feeding fed-batch bioreactor system can be recognized as 'an effective batch culture' where the term dilution rate (D), defined as F/V, can be set to zero. [Englezos and Kalogerakis, 2001] Details and examples of the evaluation of all specific rates are postponed to be illustrated in Appendix A.

***Table 3.2-4* : General expressions for evaluation of specific rates in an intermittently-feeding fed-batch bioreactor system.**

Cell balance

$$\ln X_v(t_i) - \ln X_v(t_o) = \mu[t_i - t_o]$$

Substrate balance

$$C_S(t_i) - C_S(t_o) = -r_S \int_{t_o}^{t_i} X_v(t)dt$$

Product balance

$$C_P(t_i) - C_P(t_o) = r_P \int_{t_o}^{t_i} X_v(t)dt$$

3.3 Metabolic engineering

Regarding metabolic engineering approach, there are two main constitutional aspects to be discussed; namely, narrative-based aspect and mathematical-modeling-based aspect. Detailed discussion of both aspects will be extensively covered as follows. It is to be noticed that this section focuses on only metabolic flux analysis, one field of metabolic engineering. On the other hand, the others will be concisely mentioned.

3.3.1 Narrative-based aspect

As previously mentioned in the first chapter, *INTRODUCTION*, metabolic engineering is considered as directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology. [Bailey, 1991 and Stephanopoulos *et al*, 1998] An overview insight of metabolic engineering is illustrated as in *Figure 3.3-1*.

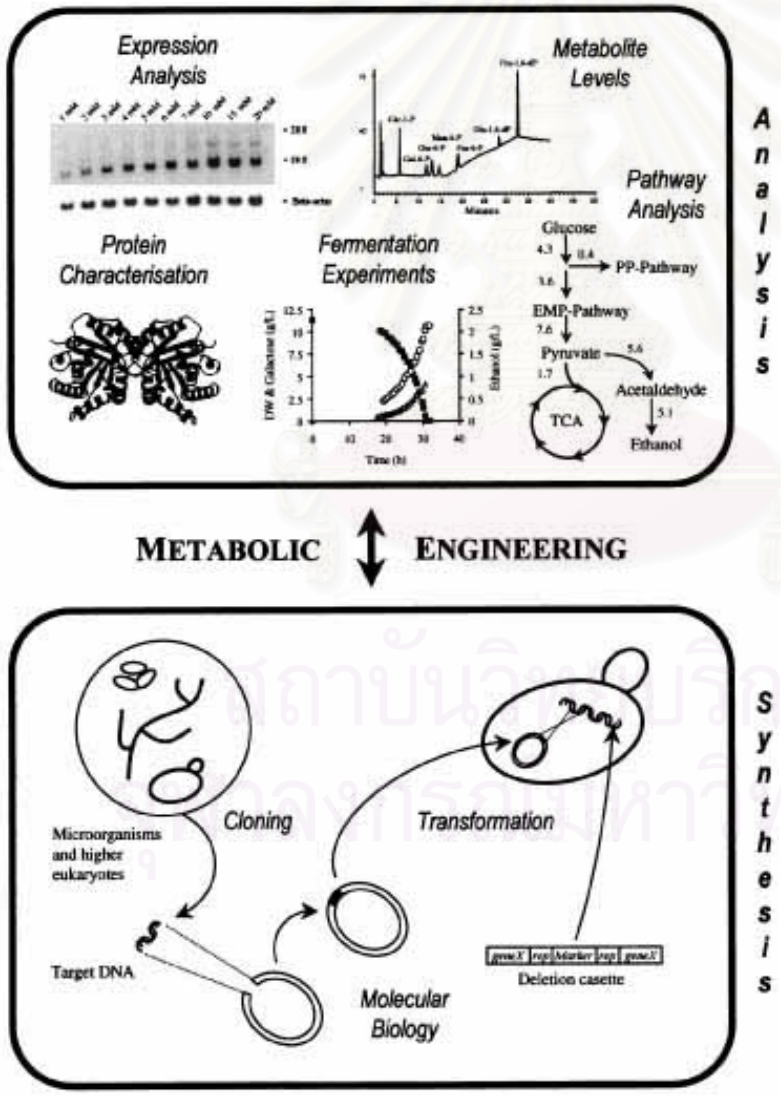


Figure 3.3-1 **: An overview of metabolic engineering.** [Ostergaard *et al*, 2000]

According to the overview illustrated in *Figure 3.3-1,* metabolic engineering consists of two important parts; namely, the analytical side of metabolic engineering and the synthesis side of metabolic engineering. The analytical side deals with the analysis of the living cell in order to identify the most promising target(s) for either genetic modification or enzymatic manipulation. While the synthesis side involves with genetic engineering of the living cell accomplishing the related construction of the living cell through either genetic modification or enzymatic manipulation. Occasionally, to achieve metabolic engineering activity, both sides of metabolic engineering inevitably contribute to each other.

As formerly stated, the most promising target for metabolic engineering can be categorized into five strategic issues; namely, extension of substrate range, improvement of productivity and yield as well as elimination of by-product, improvement of process performance, improvement of cellular properties, and extension of product range.

Focusing on the analysis side of metabolic engineering, there are three stages always interconnected with one another; namely, metabolic pathway analysis (MPA), metabolic flux analysis (MFA), and metabolic control analysis (MCA). Principally, metabolic pathway analysis deals with the acquisition of the most feasible and probable metabolic network interconnecting the specified substrate(s) and product(s). While the second, metabolic flux analysis, reveals the degree of pathway engagement in the overall metabolic process. Whilst the last, metabolic control analysis, is considered as the tool for investigating and directing the effect of any system perturbations by employing the concept of enzyme kinetics.

Metabolic pathway analysis, also known as metabolic pathway synthesis, deals with the construction of stoichiometrically consistent and thermodynamically favorable routes of biochemical reactions that meet certain specifications. [adapted from Stephanopoulos *et al*, 1998] Generally, the most common specification is the synthesis of a set of metabolic products from a designated set of substrates. Fundamentally, the elementary background on biochemistry and bioinformatics is to be inevitably perceived.

To obtain the most feasible metabolic network, systematic enumeration of all possible routes is to be carried out. With the application of computer, elaborate algorithm developed by Mavrovouniotis *et al* (1992a and 1992b), is suggested to deliberately figure out all enumerations of interest.

After performing metabolic flux analysis with the studied metabolic network, basically, the corresponding metabolic flux distribution can be obtained. In addition to quantification of pathway fluxes, metabolic flux analysis can provide additional four insights about other important cell physiological characteristics; namely, calculation of non-measured extracellular fluxes, identification of alternative pathway, calculation of maximum theoretical yield, and identification of branch point control in cellular pathway. [Stephanopoulos *et al*, 1998]

As for calculation of non-measured extracellular fluxes, flux split ratio determined from previous experiments can be used if the number of extracellular fluxes that can be measured is smaller than that which is needed to calculate the unknown intracellular fluxes.

Particularly, metabolic flux analysis can be applied to validate the existing metabolic pathway and, additionally, identify the alternative pathway that is newly-proposed. Whether the pathway is feasible depends greatly on the consistency of the material balance concept.

Based on the biochemical knowledge, important constraints on metabolic intermediates and currency metabolites are included in the calculation of maximum theoretical yield by means of metabolic flux analysis. As a result, the more reliable yield can be much more simply obtained than other approaches.

Eventually, the concept of identification of branch point control in cellular pathway can be pictorially conceived. Through comparison of changes in branch point flux split ratios resulting from changes in either different operating conditions or different mutants, the nodal

rigidity of the branch point can be assessed. Interestingly, this concept can be used for rationalizing the type of changes that will be most effective for improving product yield.

Developed from the landmark papers of Kacser and Burns (1973) as well as Heinrich and Rapoport (1974), metabolic control analysis can be considered as the tool for establishing some rigor in the mostly qualitative treatment of enzyme kinetics interactions and flux control. Furthermore, it has provided a useful framework for the systemic evaluation and description of metabolic parameters important in the control of flux. [Stephanopoulos *et al*, 1998]

In practice, the application of metabolic control analysis can be further succeeded once the rational perturbation method is selected to be studied. Specifically, the rational perturbation method can be regarded as any factors influencing on either cellular property or process performance. As for the first stated factor having influence on cellular property, genetic engineering is particularly of great contribution through the application of recombinant DNA technology. While the latter factor effecting on process performance, any process variables can be accounted into. For instance, reaction medium formulation has been extensively applied to several studies on metabolic control analysis.

Unfortunately, the related techniques to perform metabolic control analysis are imposed on measurement of either certain enzyme activity or concentration of certain intracellular metabolites. This weak point of metabolic control analysis is one of the most essential obstacles for most researches.

After performing the analytical side of metabolic engineering, certain targets are initially identified. The next step of metabolic engineering, the synthesis side, will be consecutively executed. Intensively, the following techniques of modern molecular biology can be applied; namely, amplification of any endogenous enzymes, inhibition of any enzymes, deletion of any genes, transference of any genes, and deregulation. [Advanced Course in Fermentation Technology, 2000]

Once both analytical and synthesis sides of metabolic engineering are intentionally performed for the first time. Providing that the outcome turns to be as expected, the mission can be recognized as complete operation. If not, the second trial, accompanied by the third, the forth, the fifth, and so on, is to be inevitably executed till satisfaction is met.

In conclusion, metabolic engineering contributes to the measurement and understanding of the overall flux control *in vivo*. [Stephanopoulos *et al*, 1998] And it can be clearly seen that metabolic engineering is a practical multidisciplinary synopsis of biochemistry, cell physiology, chemical engineering, genetics and molecular biology concepts as well as bioinformatics to achieve the optimal biochemical process design and control. [Advanced Course in Fermentation Technology, 2000]

As for contribution of biochemistry, the following issues of metabolic engineering can be collectively addressed; namely, rationally-proposed metabolic reaction network with supportive stoichiometry, kinetics, and regulation. With replete background of cell physiology, cellular representation of physiological state can be realized. Whereas chemical engineering is capable of providing computational tool for data analysis and system design. In addition, well-defined genetic background can be consulted through the concept of genetics and molecular biology. Finally, thanks to the newly-emerging field of bioinformatics, varieties of collections and analysis of genome/ transcriptome/ proteome and metabolome data can be conferred systemically.

3.3.2 Mathematical-modeling-based aspect

3.3.2.1 Basis equation

As for a metabolic model, the starting point focuses on the application of material balance equation of the corresponding intracellular metabolites, which will be further considered as pathway intermediates. Providing that the well-defined biochemical reaction network is rationally proposed, the following expression reveals the corresponding material balance of an intracellular metabolite i^{th} which is involved through all J reactions.

$$\frac{dx_{met,i}}{dt} = \sum_{j=1}^{J} r_{met,j} - \mu x_{met,i} \qquad \text{------- (3.3.1)}$$

Applying the above equation to all K intracellular metabolites renders the following expression in matrix notation.

$$\frac{d\overline{X}_{met}}{dt} = \overline{r}_{met} - \mu\overline{X}_{met} \qquad \text{------- (3.3.2)}$$

The left-hand side of the previous equation, $\frac{d\overline{X}_{met}}{dt}$, is accounted into the accumulation rate of the corresponding intracellular metabolites. With the crucial assumption that all intracellular metabolites are at pseudo-steady state owing to its so high turnover, the accumulation term can be set as zero as follows. It is to be noticed that this approximation appears to be valid for most intracellular metabolites based on their concentration [Zupke *et al*, 1995].

$$0 = \overline{r}_{met} - \mu\overline{X}_{met} \qquad \text{------- (3.3.3)}$$

Next, two terms of the right-hand side are to be considered. The first term, $\overline{r}_{met}$, expresses the net synthesis rate of the corresponding pathway intermediate. It represents the sum of fluxes leading into and out of a metabolic pathway. Whereas the second term, $\mu\overline{X}_{met}$, reflects the dilution of the intracellular metabolite pool owing to biomass growth. Compared with the net synthesis rate of the corresponding pathway intermediate, the dilution effect can be neglected due to its insignificantly small appearance. [Stephanopoulos *et al*, 1998] As a result, the following expression can be obtained. It is noted that the stated fluxes can be represented in the form of the relevant stoichiometric matrix and net forward reaction rate vector.

$$0 = \overline{r}_{met} = \overline{\overline{G}^{t}}\overline{v} \qquad \text{------- (3.3.4)}$$

It is noteworthy to state that the above equation is the basis equation of the metabolic model when performing metabolic flux analysis.

Then, it is recommended to partition the corresponding stoichiometric matrix and net intracellular reaction rate vector into the measured set and the rest needed to be calculated. As a result, the following expression can be obtained.

$$\overline{\overline{G^t}}\,\overline{v} \quad = \quad \overline{\overline{G_c^t}}\,\overline{v_c} + \overline{\overline{G_m^t}}\,\overline{v_m} \quad = \quad 0 \qquad \text{------- (3.3.5)}$$

3.3.2.2 Model classification

Apparently, *Equation 3.3.4* represents the *K* linear algebraic balances for the *K* metabolites with the *J* unknown reaction rates. Thus, there is a certain degree of freedom in the stated algebraic equations given below.

$$df \quad = \quad J - K \qquad \text{------- (3.3.6)}$$

Straightforwardly, according to the obtained degree of freedom, the system of algebraic equations can be classified in terms of determinacy and redundancy as summarized in *Table 3.3-1*.

Table 3.3-1 **: Classification of the algebraic system in terms of determinacy and redundancy.**
[http://www.mpi-magdeburg.mpg.de/research/project_a/pro_a5a/mfaeng/mfaprinc.html]

Term of interest	Type of system	Condition	Comment
Determinacy of non-measured rates	- Determined system	*df* = number of measured rate	- All rates are calculable.
	- Underdetermined system	*df* > number of measured rate	- Not all rates are calculable.
Redundancy of measured rates	- Redundant (overdetermined system)	*df* < number of measured rate	- Some balanceable rates exist.
	- Not redundant	*df* = number of measured rate	- No balanceable rates exists.

Fortunately, in this research, all metabolic models are the overdetermined system thanks to the fact that the number of measured rate is always greater than the degree of freedom of the model. As a result, mathematical formalism of overdetermined system will be only covered due to its relevance.

3.3.2.3 Calculation of unknown flux vector

From the basis equation, the algebraic equation system can be straightforwardly solved as follows.

Due to the non-squared term of $\overline{\overline{G_c^t}}$ in *Equation 3.3.5*, its inverse cannot be directly determined. With the application of the corresponding Moore-Penrose inverse defined as *Equation 3.3.8*, the following expression can be obtained.

$$\overline{v_c} = -\left(\overline{\overline{G_c^t}}\right)^{\#} \overline{\overline{G_m^t}}\,\overline{v_m} \qquad \text{------- (3.3.7)}$$

$$\left(\overline{\overline{G_c^t}}\right)^{\#} = -\left(\overline{\overline{G_c}}\,\overline{\overline{G_c^t}}\right)^{-1} \overline{\overline{G_c}} \qquad \text{------- (3.3.8)}$$

The above expressions can be used for calculation of unknown flux vector, $\overline{v_c}$. In spite of their simplicity and straightforwardness, they are not popularly applied in metabolic flux analysis. Because they neglect errors from measurement noises. As can be easily seen, $\overline{v_c}$ is computed directly from $\overline{v_m}$, which may be a bit erroneous.

3.3.2.4 Data reconciliation

Instead of calculation of $\overline{v_c}$ based on only $\overline{v_m}$, Tsai and Lee (1988) proposed a novel procedure to obtain better estimates for both non-measured and measured fluxes. This procedure is carried out to overcome the inconsistency resulting from any noises accompanied by measurement variation. By this means, $\overline{v_c}$ is computed from reconciled $\overline{v_m}$.

First, they reformulated *Equation 3.3.5*, to the following expression by introducing a newly-defined matrix $\overline{\overline{T}}$.

$$\begin{pmatrix} \overline{v}_m \\ \overline{0} \end{pmatrix} = \begin{pmatrix} \overline{\overline{I}} & \overline{\overline{0}} \\ \overline{\overline{G^t}} & \end{pmatrix} \begin{pmatrix} \overline{v}_m \\ \overline{v}_c \end{pmatrix} = \overline{\overline{T}}\,\overline{v} \qquad \text{------- (3.3.9)}$$

Apparently, the first row of the above expression reflects the equality of $\overline{v_m}$ with the contribution of the identity matrix and zero matrix. While the second row is simply identical to *Equation 3.3.4*.

If the measurement errors are distributed normally with a mean value of zero and a variance-covariance matrix equal to $\overline{\overline{F}}$, the new estimates for the elements of $\overline{v_1}$, $\tilde{v}_1$, can be obtained as follows. [Madron *et al*, 1977]

$$\tilde{v} = \left(\overline{\overline{T^t}}\,\overline{\overline{F^{-1}}}\,\overline{\overline{T}}\right)^{-1} \overline{\overline{T^t}}\,\overline{\overline{F^{-1}}} \begin{pmatrix} \overline{v}_m \\ \overline{0} \end{pmatrix} \qquad \text{------- (3.3.10)}$$

Unfortunately, if the variance and covariance information is not available, the new estimates for the elements of $\overline{v_1}$, $\tilde{v}_1$, can be computed with the application of the least squares estimation as follows.

$$\tilde{v} = \left(\overline{\overline{T}}^t \overline{\overline{T}}\right)^{-1} \overline{\overline{T}}^t \begin{pmatrix} \overline{v}_m \\ \overline{0} \end{pmatrix} \qquad \text{------- (3.3.11)}$$

In this research, *Equation 3.3.11* is used for the estimation of both non-measured and measured fluxes. Those estimated fluxes are quite more rational than those directly obtained from *Equation 3.3.7* since the concept of data reconciliation is attentively taken into account.

3.3.2.5 Sensitivity analysis

Importantly, before commencing metabolic flux analysis, the proposed metabolic model is to be initially verified and analyzed. There are two approaches to perform sensitivity analysis of the proposed model. The first one can be considered as the model sensitivity while the latter is essentially according to flux sensitivity.

As for model sensitivity, the so-called condition number of an algebraic system can be used as the indicator for identifying whether the system is ill-conditioned. Providing that the calculated condition number is significantly large, the system is likely to be an ill-conditioned system.

The corresponding condition number of proposed metabolic model can be obtained from the following expression.

$$C\left(\overline{\overline{T}}\right) = \left\| \overline{\overline{T}} \right\| \left\| \left(\overline{\overline{T}}\right)^{\#} \right\| \qquad \text{------- (3.3.12)}$$

It is to be noticed that in this research we compute the condition number of matrix $\overline{\overline{T}}$ instead of $\overline{\overline{G^t}}$. Because when performing flux calculation, we apply *Equation 3.3.9*, which is apparently not *Equation 3.3.5*, to obtain flux estimates.

The magnitude of the condition number provides important information on requirements for the accuracy of the measured fluxes. This implies that the measurements have to be carried out with the precision that carries the same number of digits as there are in the condition number. Because fermentation rates can be rarely quantified with precision greater than two significant digits. Specifically, it is suggested that the condition number of well-conditioned system be in the order of hundreds. [Stephanopoulos et al, 1998]

Turning to the second approach, flux sensitivity can be simply accomplished by rearranging *Equation 3.3.7* into the following expression.

$$\frac{\partial \overline{v}_c}{\partial \overline{v}_m} = -\left(\overline{\overline{G}}_c^t\right)^{\#} \overline{\overline{G}}_m^t \qquad \text{------- (3.3.13)}$$

In the above equation, the element in the *j*th row and *i*th column reports the sensitivity of the *j*th flux, which is calculated, with respect to variations in the measurement of the *i*th flux. Thus, it can provide additional information on the sensitivity of the system with respect to variations in the measured fluxes.

In conclusion, there are mainly six aspects in metabolic flux analysis of structured model already discussed. *Table 3.3-2* summarizes these six basic aspects for the sake of a quick reference.

***Table 3.3-2* : Summary of basic concept of structured model.**

Aspect	Comment
Employed material balances	Material balances of intracellular metabolites
Basis expression	$\begin{pmatrix} \bar{v}_m \\ \bar{0} \end{pmatrix} = \begin{pmatrix} \bar{\bar{I}} & \bar{\bar{0}} \\ \bar{\bar{G}}^t \end{pmatrix} \begin{pmatrix} \bar{v}_m \\ \bar{v}_c \end{pmatrix} = \bar{\bar{T}}\bar{v}$
Degree of freedom of the model	$J - K$
Calculation of non-measured flux vector and reconciliation of measured flux vector	$\tilde{v} = \left(\bar{\bar{T}}^t \bar{\bar{T}}\right)^{-1} \bar{\bar{T}}^t \begin{pmatrix} \bar{v}_m \\ \bar{0} \end{pmatrix}$
Determination of condition number (model sensitivity)	$C\left(\bar{\bar{T}}\right) = \left\|\bar{\bar{T}}\right\| \left\|\left(\bar{\bar{T}}\right)^{\#}\right\|$
Flux sensitivity	$\frac{\partial \bar{v}_c}{\partial \bar{v}_m} = -\left(\bar{\bar{G}}_c^t\right)^{\#} \bar{\bar{G}}_m^t$

CHAPTER 4
DEVELOPMENT OF METABOLIC NETWORKS

4.1 Biochemistry of relevance
4.2 Reviews of metabolic networks for poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosyntheses
4.3 Crucial hypotheses
4.4 Metabolic networks of interest
4.5 Mathematically-feasible metabolic networks

Primarily, the first task of metabolic flux analysis is a construction of a metabolic network. To be fruitful or worthless, any suggestions drawing from metabolic flux analysis are susceptible to that challenging task. Therefore, a proposed metabolic network demands such a solid development.

As a matter of fact, there are two consecutive stages concerning development of metabolic network. The first stage involves mainly the concept of biochemistry, while the second stage concerns mostly mathematical analysis.

Regarding as one of the most difficult chore, assuredly for a chemical engineer, biochemistry background is to be intensively perceived. Due to information on the bacterial strain of interest has been scarcely reported, the relating, presumably, are exploited instead. Accordingly, it will be summarized in this chapter as section 4.1 and 4.2

The exertion of biochemistry information of other microbe is not the only presumption. Skeptically, a few more are to be inclusively acclaimed. All assumptions dealing with development of our metabolic networks are summarized as section 4.3

In the end of this chapter, metabolic networks of interest are rationally proposed as appeared in section 4.4. Honestly, they are considered to be the most biochemically-feasible and, in the same time, mathematically-solvable ones. The latter characteristics of the metabolic network of interest are crucial and demanding. To validate their solvability, section 4.5 is finally attached to this chapter.

Eventually, the metabolic networks of interest will be further employed in the next chapter, metabolic flux analysis.

4.1 Biochemistry of relevance

Apparently, there are mainly nine categories of cellular metabolism involve in polyhydroxyalkanoate (PHA) biosynthesis in *Ralstonia eutropha* NCIMB 11599; namely, glucose catabolism, propionic acid catabolism, tricarboxylic acid (TCA) cycle, PHA formation route, ammonium assimilation, residual biomass formation, electron transport and oxidative phosphorylation, transhydrogenation, and anaplerotic pathway. This topic will summarize substantial researches on biochemistry of relevance.

4.1.1 Glucose catabolism

As for a carbohydrate catabolism, the wild-type strain *Ralstonia eutropha* H16 is known to catabolize fructose or gluconate to pyruvate via the Entner Doudoroff (ED) pathway. [Gottschalk et al., 1964; De Ley et al., 1970; all reviewed in Conway, 1992] Likewise, the glucose-utilizing mutant *Ralstonia eutropha* NCIMB 11599, the strain of interest, was evidenced to degrade glucose to pyruvate through the ED pathway after the facilitated diffusion uptaking system. [Schlegel and Gottschalk, 1965; K\nig et al., 1969; Pries et al., 1990; all reviewed in Joo, 1998]

Further evidence on the importance of the ED pathway is the inability of certain mutants of *R. eutropha* that possess decreased levels of the ED pathway enzymes to grow on fructose [Bowien and Schlegel, 1972a] and the excretion of 2-keto-3-deoxy-6-phosphogluconate (KDPG) by a mutant that lacks KDPG aldolase [Bowien and Schlegel, 1972b].

Detailed study on the operating mode of the ED pathway was conducted by Reutz et al. (1982). They reported that phosphoglycerate mutase-deficient mutants of *R. eutropha* lost the ability to grow on certain gluconeogenic substrates; succinate, pyruvate, acetate, and citrate. But the mutants were still able to grow, but at a slower rate than the wild type, on fructose or gluconate. This meant the mutants could completely degrade fructose or gluconate to pyruvate by a special metabolic process to bypass the phosphoglycerate mutase lesion. This suggested that in *R. eutropha* the ED pathway operates in a cyclic mode. Interestingly, the cyclic mode of the ED pathway mediates itself as a recycling route of glyceraldehyde-3-phosphate (GA3P) back into the upper part of the ED pathway. (See *Figure 3.1-7* for a schematic pathway of cyclic mode of ED pathway)

They also concluded that phosphoglycerate mutase is dispensable in glycolysis of carbohydrate; though, its gluconeogenic function is necessary. It implies KDPG aldolase could not serve as a gluconeogenic enzyme despite its reversibility. This implication is further realized in one part of our work, specifically, when constructing a metabolic network that describes the metabolic behavior of *R. eutropha* growing on propionic acid as a sole carbon and energy source.

In addition, versatilely, the cyclic mode of the ED pathway can serve not only glycolytic but also gluconeogenic function as well. This can be easily supported by the fact that it contains all key enzymes that could contribute for gluconeogenesis. [Reutz et al., 1982; Schobert and Bowien, 1984; Br™mer and Steinb\chel, 2002]

The glycolytic Embden-Meyerhof-Parnas (EMP) pathway, which is commonly found in various microorganisms for carbohydrate degradation, is not believed to operate in *R. eutropha*. (See *Figure 3.1-5* for a schematic pathway of EMP pathway) This was evidenced by a novel research of Steinb\chel who found that *R. eutropha* is devoid of 6-phosphofructokinase, which is one of the key enzymes of the EMP pathway. [Steinb\chel, 1987]

Unlike other ordinary bacteria, *R. eutropha* growing on glucose lacks another common enzyme, 6-phosphogluconate dehydrogenase. [Schlegel and Gottschalk, 1965] Particularly, the stated enzyme acts as a gateway of the pentose-phosphate (PP) pathway. [White, 2000] The PP pathway, as a result, is seemingly not fully operated in *R. eutropha*. This was also stated in the Ph.D. thesis of Joo (1998)

However, certain intermediary tetrose and pentose sugars are still needed for biosyntheses of macromolecular cellular constituents. [Personal communication with Bowien, 2003] Cortassa et al. (1995) proposed a modified route of the PP pathway in which these intermediary tetrose and pentose sugars could be synthesized. Saying, the route consisted of only the transaldolase and transketolase rearrangement reactions. With the above simplification, Shi et al. (1997) could adopt the PP pathway as an anabolic function in their metabolic flux analysis research. (See r_{23} and r_{24} in *Figure 4.2-7* for a schematic pathway of modified pattern of PP pathway)

4.1.2 Propionic acid catabolism

Regarding the catabolism of propionic acid, so far investigated, there has been no conclusion report on the strain of interest. Fortunately, there has been only the evidence of the neighboring strain.

Primarily, it was believed that propionic acid might be degraded via various routes; for instances, the α-oxidation, β-oxidation, α-carboxylation or so-called as methylmalonyl-CoA pathway, reductive carboxylation, and Claisen condensation. [all reviewed in Textor et al., 1997; Tsang et al., 1998; Horswill and Escalante-Semerena, 1999; Luttik et al., 2000]

However, recently, the 2-methylcitric acid (MCA) cycle, one form of the Claisen condensation, was proposed to operate in *Ralstonia eutropha* HF39 growing on propionic acid. [Br™mer and Steinb⎫chel, 2001] Interestingly, the MCA cycle was evidenced to operate in two PHA-biosynthesizing bacteria; recombinant *Escherichia coli* [Valentin et al., 2000] and *Burkholderia sacchari* IPT101 T [Br™mer et al., 2002]. (See *Figure 3.1-8* for a schematic pathway of MCA cycle) Prominently, these reports were confirmed by the physiological, enzymic, and genomic evidence.

4.1.3 Tricarboxylic acid (TCA) cycle

Like ordinary microbes, the tricarboxylic acid (TCA) cycle is also present in *R. eutropha*. The existence of a complete TCA cycle was confirmed by the radiorespirometric study. Discovery of all enzymes participating in the TCA cycle was reported by Tr⎫per (1965) and Kelly (1971). (See *Figure 3.1-9* for a schematic pathway of TCA cycle)

It is to be noticed that although *R. eutropha* possesses both NAD- and NADP-linked isocitrate dehydrogenase activities, the latter was only of interest. Because the latter activity is severalfold higher [Charles, 1970; Glaeser and Schlegel, 1972a; Matin and Rittenberg, 1970; McFadden and Howes, 1962; Tr⎫per, 1965; all reviewed in Matin, 1978] and also serves as a primary role in energy generation. [Glaeser and Schlegel, 1972b; reviewed in Matin, 1978]

4.1.4 Polyhydroxyalkanoate formation route

Details on the biochemistry of 3HB and 3HV formation routes are already summarized in Chapter 2. As a result, it will not be repeatedly cited in this topic. However, a crucial issue associated with its nature is to be stated and augmented when constructing the metabolic networks of interest.

Actually, biosynthesis and degradation of PHA are mutually operated in a cyclic fashion. Nonetheless, metabolic flux analysis cannot differentiate both when performing flux calculation. Specifically, the net action, that is the net biosynthesis in this case, is presumably on an account. This simplification was also applied in substantial researches on metabolic flux analysis. [Shi et al., 1997; Shi et al., 1999; Wong et al., 1999; Yu and Wang, 2001; and Chanprateep, 2001]

4.1.5 Ammonium assimilation

Like all other hydrogen bacteria, *R. eutropha* is able to utilize ammonium as an essential nitrogen source. Ammonium is mainly incorporated into glutamate or glutamine, and in turn these amino acids serve as the main precursor for the synthesis of other cellular organic nitrogen compounds. [Harder and Dijkhuizen, 1980 reviewed in Joo, 1998]

So far investigated, conclusive information for ammonium assimilation in *R. eutropha* is not currently available. But it was believed that ammonium assimilation in this bacterium is carried out with two mechanisms, which operate differently according to extracellular concentrations of ammonium. [Bowien and Schlegel, 1981] (See *Figure 3.1-10* for a schematic pathway of both routes)

When the concentration of ammonium in the environment of the *R. eutropha* cells is high, it is taken up by facilitated diffusion and subsequently assimilated by NADPH-linked glutamate dehydrogenase (GDH). At its low concentration, ammonium is believed to be taken up by an active transport system, and in this circumstance, glutamine synthetase (GS) and glutamate synthase (GOGAT) are responsible for assimilation of ammonium into amino acids. [Bowien and Schlegel, 1981; Harder and Dijkhuizen, 1983]

Heinzle and Lafferty (1980) also suggested that in *R. eutropha* ammonium ion is assimilated through these two mechanisms with different saturation constants (K_m), that is 1.0 and 0.1 g/l, respectively.

4.1.6 Residual biomass formation

As a matter of fact, formation of residual biomass is considerably complicated. The process starts as soon as nitrogen source is assimilated into the cell. After that, hundreds of reactions subsequently take place. Unfortunately, this complete information has not been available for *Ralstonia eutropha*.

Thanks to the study of Shi et al in 1997, we could adopt a biochemical reaction used for describing the formation of all macromolecular cellular constituents. Specifically, just a simple lump reaction was used instead of a fully-characterized reaction set. The succeeding paragraph will reveal their rationale to develop the stated lump reaction.

Since it is currently accepted that the metabolic routes from key precursor metabolites to small molecules are common to a wide variety of organisms. They first calculated the produced key precursor metabolites from these routes by using the information of *Escherichia coli*, proposed by Min et al. (1996). Then the precursor metabolite requirement for the synthesis of monomers in *E. coli* developed by Holms (1986) was adopted as a basis

for the next step. Finally, residual biomass formation was represented as a lump reaction, where precursor yield coefficients were corrected to match the residual biomass formula of *R. eutropha*, that is $C_4H_{6.9}N_{0.98}O_{1.63}$ (with 5% ash), which was taken from Ampe et al. (1996)

4.1.7 Electron transport and oxidative phosphorylation

Likewise, information on electron transport and oxidative phosphorylation is lack for *Ralstonia eutropha*. But for most prokaryotes, the theoretical stoichiometry of oxidative phosphorylation, the so-called P/O ratio, is 2 moles of ATP synthesized for each mole of NADH2 oxidized. [White, 2000] Since only two sites appear to translocate protons in them. However, due to incomplete coupling of the oxidation and phosphorylation processes, the operational P/O ratio is much below the theoretical values.

In constructing the metabolic networks, Shi et al. (1997) made another valuable assumption on this metabolic process. They postulated that the maximum 2 moles of ATP could be synthesized for each mole of NADH2 oxidized. In addition, 1 mole of ATP could be synthesized for each mole of FADH2 oxidized.

To account for maintenance requirements and futile cycles, the dissipation of excess ATP is to be included in the metabolic networks. It is simply included to provide an estimate of excess energy availability and possible energy limitations during the fermentation process. [Stephanopoulos et al., 1998]

For the sake of simplicity, we also apply these stoichiometries in our metabolic networks of interest.

4.1.8 Transhydrogenation

Commonly, transhydrogenation is regarding as a reversible conversion between two key currency metabolites, that is NADH and NADPH. Nonetheless, its physiological role has not been clearly understood yet. [Hoek and Rydstr\m, 1988] Moreover, its presence in *R. eutropha* is somewhat contradictory.

A previous research group postulated that transhydrogenase might exist in *R. eutropha* during the balanced growth. Its direction is in the NADPH generation by the ATP-requiring reduction of $NADP^+$. [Jones et al., 1975 reviewed in Joo, 1998]

However, a recent study showed that a crude extract of *R. eutropha* grown on glucose under nitrogen limitation exerted only a trace level of the transhydrogenase activity. [Lee et al., 1995 reviewed in Joo, 1998]

Another supporting idea for the latter is proposed by Hoek and Rydstr\m (1988). They suggested that in general, if transhydrogenase is present, it is not likely to play an important role in the overall cellular metabolism under normal growth conditions.

Unfortunately, these reports are not available for us to see the exact cultivation conditions. As a result, we are not sure whether in our cases it should be incorporated in the metabolic networks of interest. This consideration will be covered in detail in the succeeding topic.

4.1.9 Anaplerotic pathway

To replenish the biosynthesis of oxaloacetate (OAA), two remarkable publications suggested that anaplerotic pathway in *Ralstonia eutropha*. The one was conducted by Schobert and Bowien (1984) and the other was by Br™mer and Steinb⏐chel (2002). The previous research group drew such conclusion from enzymic and physiological analysis of phosphoenolpyruvate (PEP) synthetase-deficient mutant of *R. eutropha* H16. While the latter did so with a TN5-induced malate dehydrogenase-deficient mutant of *R. eutropha* HF39.

Surprisingly, these two studies concurrently suggested the same anaplerotic pathways for various carbon sources. Accordingly, depending upon the growth substrate, diversed patterns are confirmatively proposed. (see *Figure 4.2-1* and *Figure 4.2-2* for a schematic pathway for anaplerotic pathways)

If the carbon source of interest is fructose or gluconate, PEP carboxykinase serves as the only anaplerotic route to replenish OAA. This is evidenced by the fact that growth on fructose or gluconate was not affected by the enzyme defect.

When growing on pyruvate or lactate, three-carbon containing substrate, the anaplerotic pathway relies on the consecutive function of PEP synthetase and PEP carboxykinase. Neither the glyoxylate bypass enzymes, such as isocitrate lyase and malate synthase, nor the NADPH-linked malic enzyme can support the anaplerotic production of OAA. Furthermore, although the reaction catalyzed by the malic enzyme is reversible, the physiological importance of the *R. eutropha* malic enzyme is gluconeogenically in the formation of pyruvate from malate. This is evidenced by the very low activity of isocitrate lyase in pyruvate-grown *R. eutropha*. Additionally, the mutants were totally unable to grow on pyruvate or lactate.

On the other hand, two alternative pathways are present if it is grown on any four-carbon dicarboxylic acid, like malate or succinate. Besides decarboxylation of OAA by PEP carboxykinase, the consecutive action of NADPH-linked malic enzyme and PEP synthetase can also accomplish this synthesis. Since the mutants grew slower than the wild type on these substrates.

There is a conflict evidence for the presence of other C3-carboxylating enzymes. As the first investigation for the anaplerotic pathway in *R. eutropha*, Frings and Schlegel (1971) reported that *R. eutropha* possesses both PEP carboxylase and PEP carboxykinase but lacks pyruvate carboxylase. But later work argued the presence of the PEP carboxylase. [Schobert and Bowien, 1984]

Unfortunately, there is no evidence for the anaplerotic pathway in propionic acid-grown *R. eutropha*. There have been merely two novel works on the relating microbes.

Textor et al. (1997) concluded that in *Escherichia coli* K12 OAA is regenerated mainly through the glyoxylate shunt. They confirmed by the isotope labeling studies and also by the analysis of glyoxylate shunt mutants that could not grow on propionate.

Unlike the previous study, Horswill and Escalante-Semerena (1999) proposed PEP carboxylase, not glyoxylate shunt, might serve as an only OAA regeneration in *Salmonella typhimurium* LT2. Since PEP synthetase-deficient mutants failed to utilize propionate while glyoxylate shunt mutants grew well on propionate.

4.2 Reviews of metabolic networks for poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosyntheses

Universally, there have been several explorations on PHA biosyntheses in various microbes and even certain transgenic plants. Most of them merely gaze at a mechanism of the biosynthesis step not at a whole picture of cellular metabolism. To accomplish a task on metabolic flux analysis, however, that 'whole picture' is to be incorporated in a research process. This section will review certain helpful publications in regard to development of 'a whole picture' of metabolic networks describing the biosyntheses of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate).

Schobert and Bowien (1984) summarized a metabolic network narrating central carbon metabolism in *R. eutropha* H16 growing on various carbon sources. As shown in *Figure 4.2-1*, the network encompassed cyclic mode of ED pathway, Calvin cycle, TCA cycle, and anaplerotic pathways. Two remarkable aspects can be drawn out from their findings.

The first one is the inclusion of Calvin cycle in the network. This implied fascinating mixotrophic capability of *R. eutropha*, say it could utilize the assimilated carbon dioxide at the same time it degrades any ordinary carbohydrates. However, this issue will not be taken into account in our work. The reason of this will be exposed in the next section of this chapter.

Another notable prospect is its revelation of the possible anaplerotic pathways in *R. eutropha* as well as a dual physiological function of PEP carboxykinase. Depending upon the growth substrate, diversed patterns are confirmatively proposed. Detail of this finding has already been mentioned in the previous section. As a matter of fact, the construction of our metabolic networks relies heavily on their suggestions.

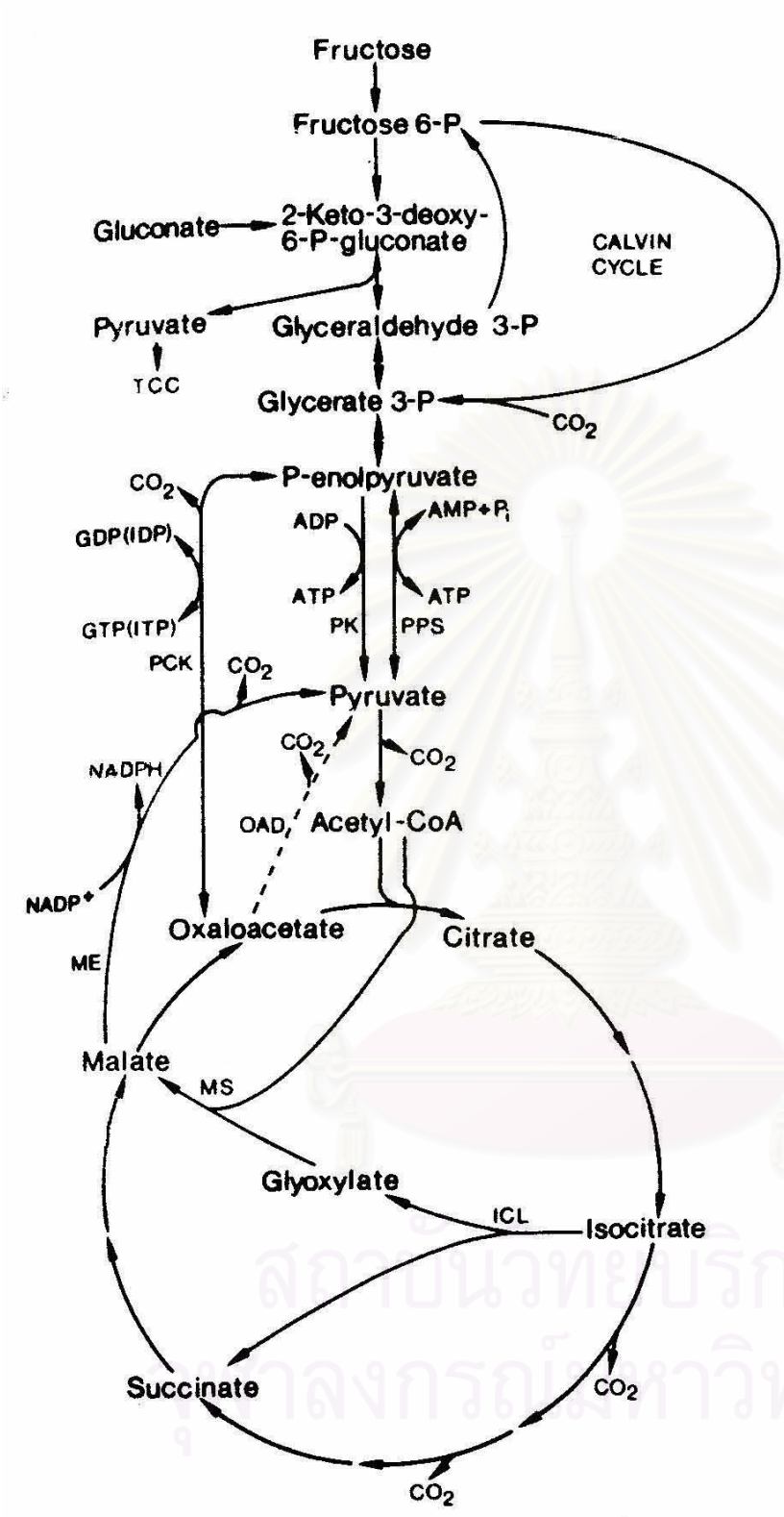


Figure 4.2-1: Metabolic network describing a central metabolism of *Ralstonia eutropha* H16 as proposed by Schobert and Bowien (1984). Abbreviations: PCK, PEP carboxykinase; PK, pyruvate kinase; PPS, PEP synthetase; ME, malic enzyme; OAD, oxaloacetate decarboxylase; ICL, isocitrate lyase; MS, malate synthase; TCC, tricarboxylic acid cycle. The dashed line indicates the possible existence of a distinct oxaloacetate decarboxylase in this organism.

Following the study of Schobert and Bowien (1984), an elegant work of Br™mer and Steinb⎪chel (2002) affirmed lots of the former suggestions. The full map of central metabolism in *R. eutropha* HF39 growing on a carbohydrate or an organic acid was finally adopted. As shown in *Figure 4.2-2*, the central metabolism embraced ED pathway, TCA cycle, and MCA cycle. In addition, the possible anaplerotic routes as suggested by Schobert and Bowien (1984) were also included in the network.

Seemingly, their proposed network is only applicable to a single-substrate system, specifically, fructose, pyruvate, various tricarboxylic acid intermediates, glyoxylic acid, acetate, propionate, or levulinate. Still, it is considerably appealing whether it could be apt with our work where a two-substrate system is concerned.

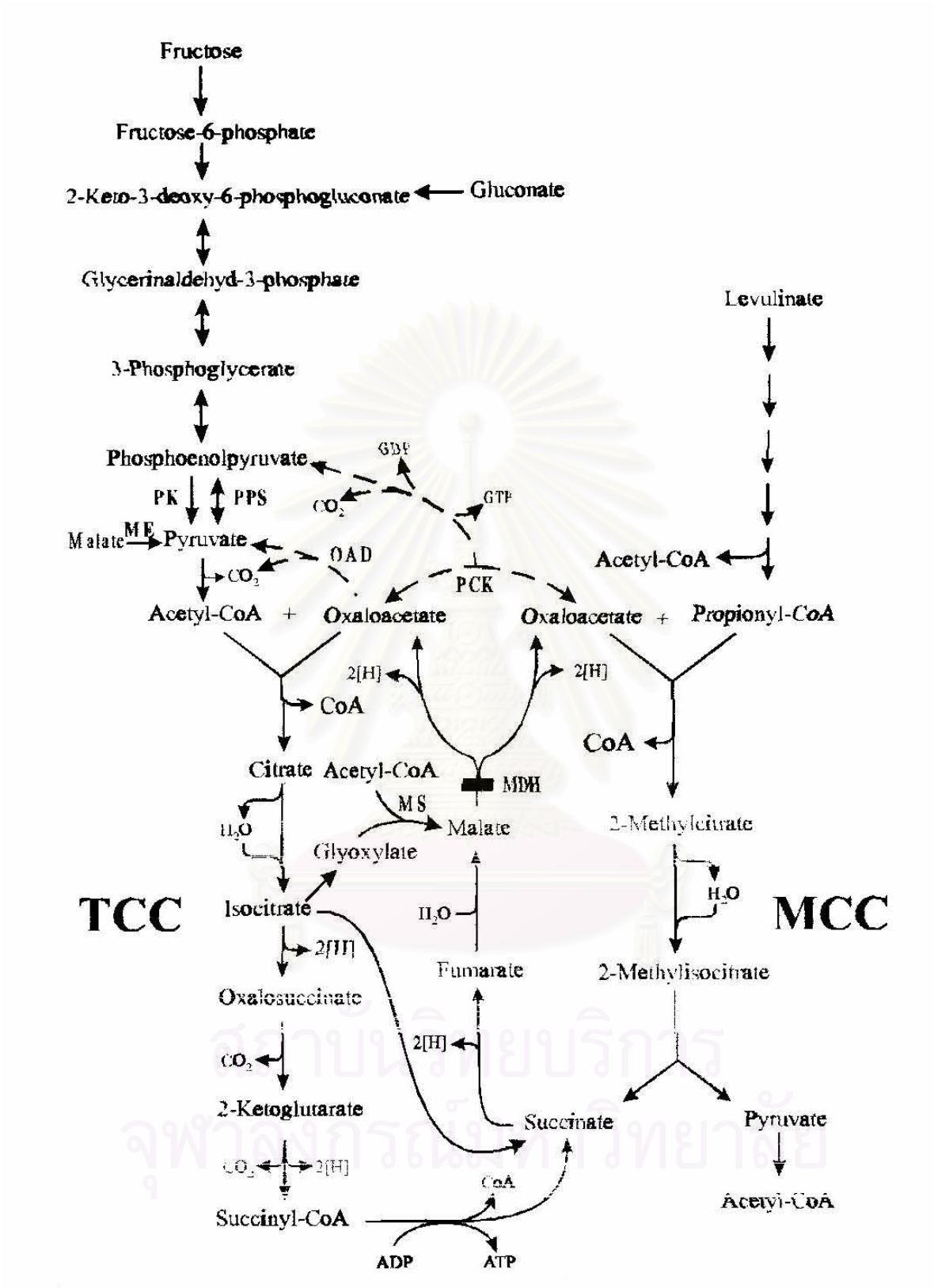


Figure 4.2-2: Metabolic network describing a central metabolism of *Ralstonia eutropha* HF39 as proposed by Br™mer and Steinb⎜chel (2002). Abbreviations: PCK, PEP carboxykinase; PK, pyruvate kinase; PPS, PEP synthetase; ME, malic enzyme; OAD, oxaloacetate decarboxylase; MDH, malate dehydrogenase; MS, malate synthase; TCC, tricarboxylic acid cycle; MCC, 2-methylcitric acid cycle.

Thanks to a novel work of Horswill and Escalante-Semerena (1999), we could adopt an outline of a metabolic network dealing with MCA cycle. In spite of the fact that the network is used for propionate metabolism in *Salmonella typhimurium* LT2, their work is still attractive since it is the only one regarding the presence of MCA cycle.

As illustrated in *Figure 4.2-3*, their network consisted of MCA cycle, TCA cycle, gluconeogenesis, and anaplerotic pathway. They roughly suggested the connection among them. Pyruvate obtaining from MCA cycle is further oxidized through TCA cycle for energy generation or converted to PEP through gluconeogenic pathway. In addition, OAA could be regenerated by the action of PEP carboxylase, the only possible anaplerotic route.

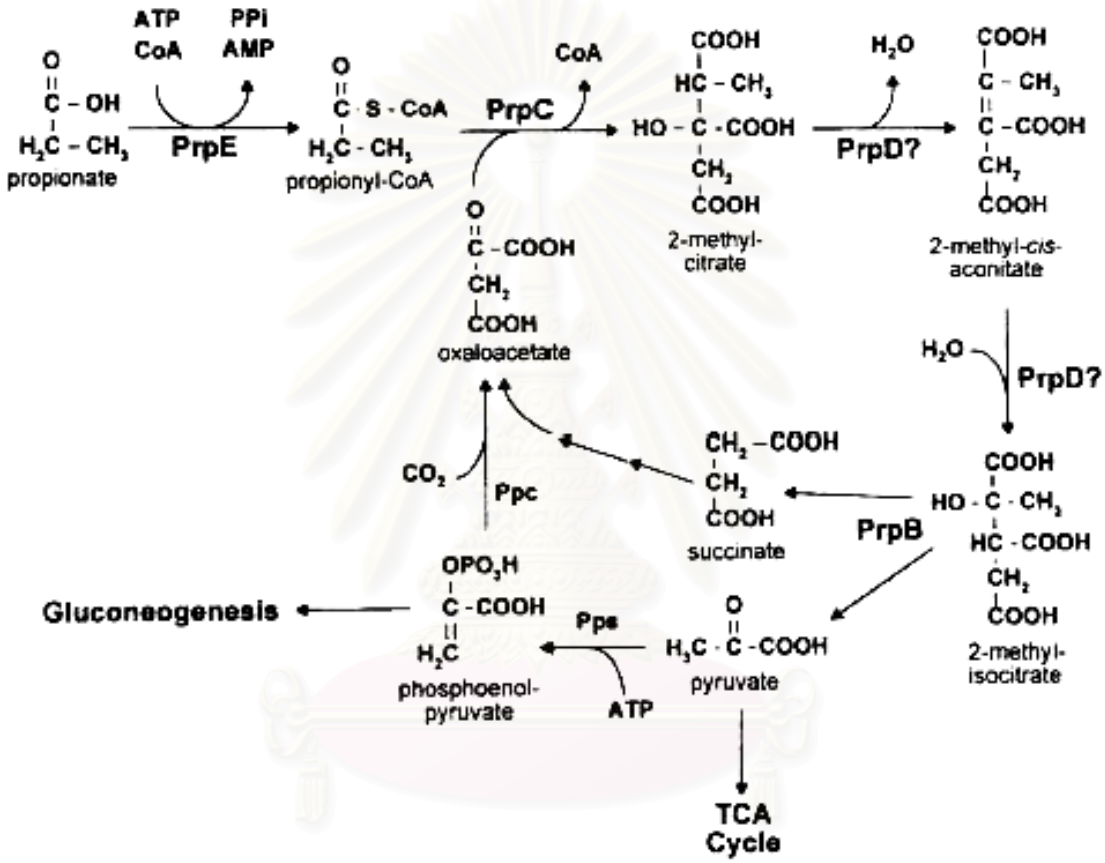


Figure 4.2-3: Metabolic network describing a central metabolism of *Salmonella typhimurium* LT2 as proposed by Horswill and Escalante-Semerena (1999). Abbreviations: Ppc, PEP carboxylase; Pps, PEP synthetase.

Moving to a bigger point of view, Wong et al. (1999) developed a metabolic network depicting P(3HB) biosynthesis in recombinant *E. coli* growing on glucose as a sole carbon and energy source. As illustrated in *Figure 4.2-4*, the network contained only 12 fluxes relating to lumped reactions of glycolysis, PHB biosynthesis, certain fermentative pathways, and energy generation and reducing power regeneration.

Since their developed metabolic network was further employed for the sake of simple metabolic flux analysis. It is substantially different from ours. But a critical point still stands from reviewing their work. These fermentative pathways played an important role in the microbe growing under oxygen-limited condition. This clue reflects an urgent need to establish one crucial hypothesis in our work. Assuredly, this will be covered in the next section.

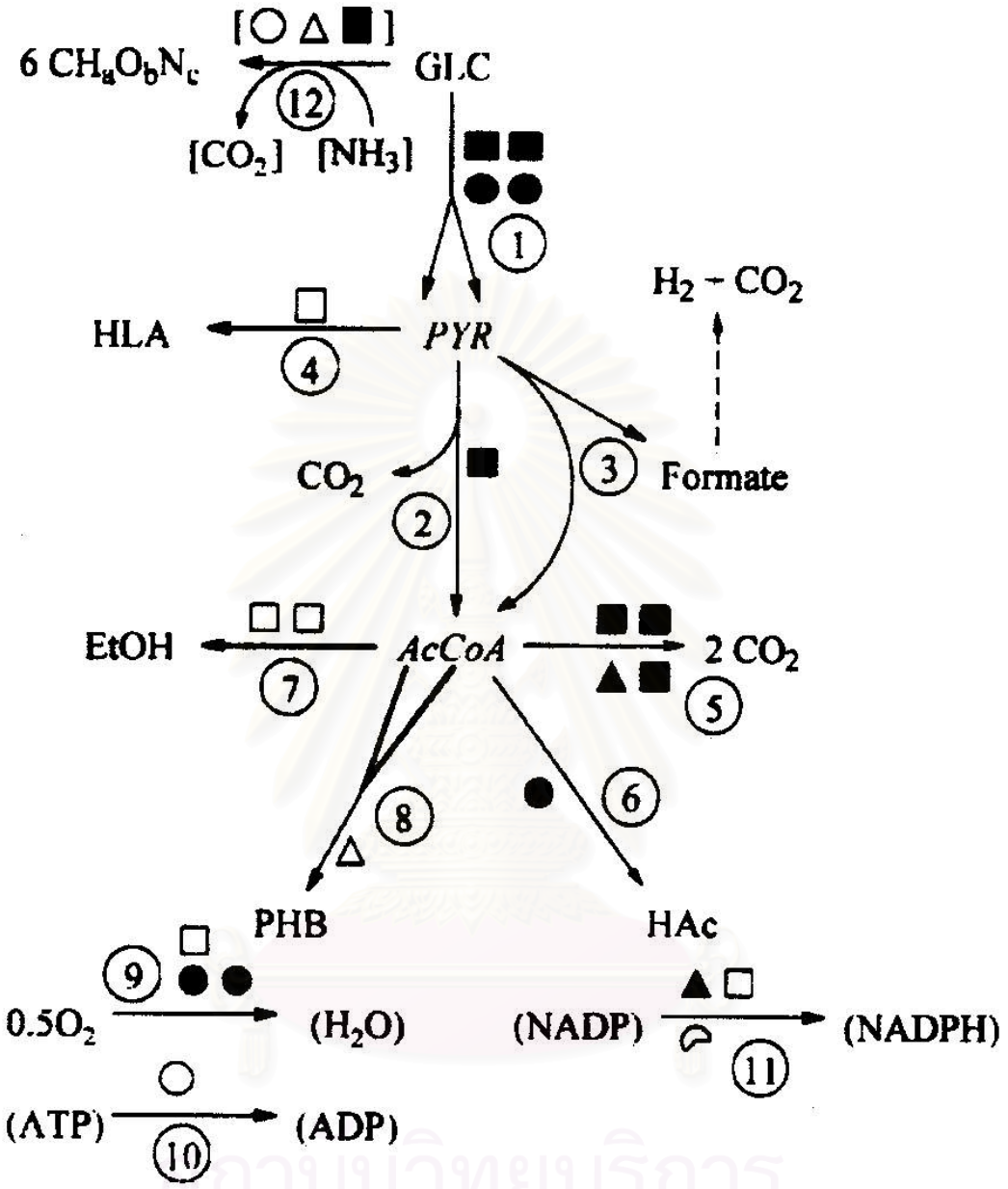


Figure 4.2-4: Metabolic networks for simple metabolic flux analysis of P(3HB) biosynthesis in recombinant *Escherichia coli* growing on glucose as a sole carbon and energy source as proposed by Wong et al. (1999).

Another simple metabolic network was developed by Yu and Wang (2001) to explain the metabolic behavior of *R. eutropha* H16 growing on acetic acid as a sole carbon and energy source. As shown in *Figure 4.2-5*, they focused on only key fluxes representing the overall mechanism of acetate uptake, AcCoA formation, TCA cycle, P(3HB) formation, and residual biomass formation.

Actually, it is somewhat dubious whether the network is developed for a metabolic flux analysis. Since except for AcCoA formation flux, the rest is essentially the extracellular rates that could easily link to the corresponding kinetic parameters. Nevertheless, it is worthwhile citing as a result of its outstanding issues, which will later be claimed in our work, acquired from the metabolic flux analysis.

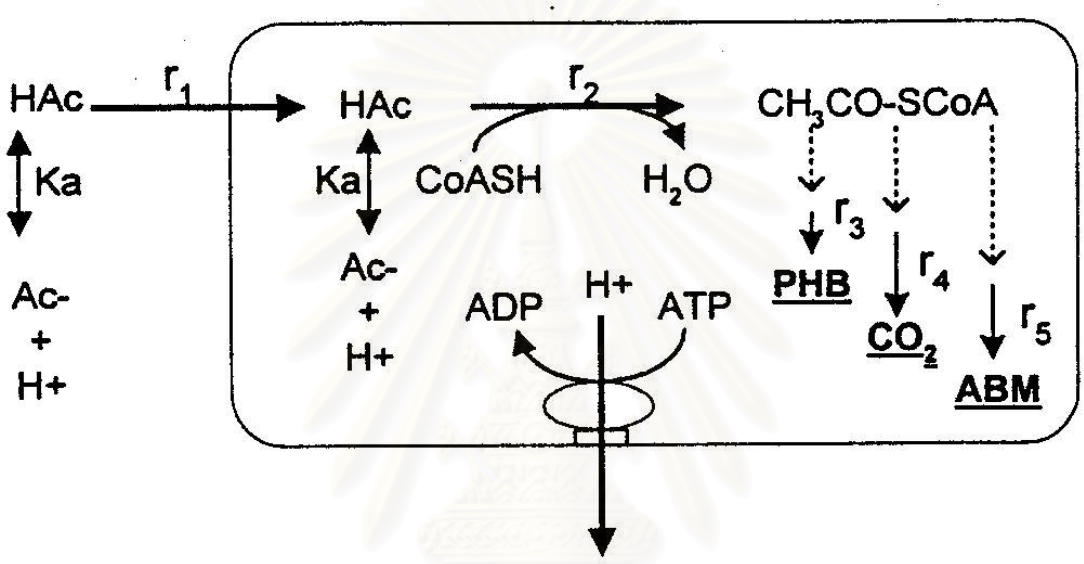


Figure 4.2-5: Metabolic networks for simple metabolic flux analysis of P(3HB) biosynthesis in *Ralstonia eutropha* H16 growing on acetate as a sole carbon and energy source as proposed by Yu and Wang (2001). Abbreviations: HAc, acetic acid; ABM, active biomass.

Similar to the work of Yu and Wang (2001), Chanprateep et al. (2001) also developed a simple metabolic network to support their experimental results. The network was applicable to P(3HB-*co*-3HV) biosynthesis in *Paracoccus denitrificans* ATCC 17741 growing on a mixture of ethanol and n-pentanol. As shown in *Figure 4.2-6*, it consisted of simple breakdown of ethanol and n-pentanol, TCA cycle, 3HB and 3HV formation routes, and possible decarboxylation of KetVaCoA to AcCoA.

In general, this work is profoundly interesting. Because it is the only one that used for describing the metabolic behavior of a microbe growing on a mixture of two carbon sources. However, it is still just a simple one that confines itself for certain objectives not for ‘a whole picture’ of delicate cellular metabolism.

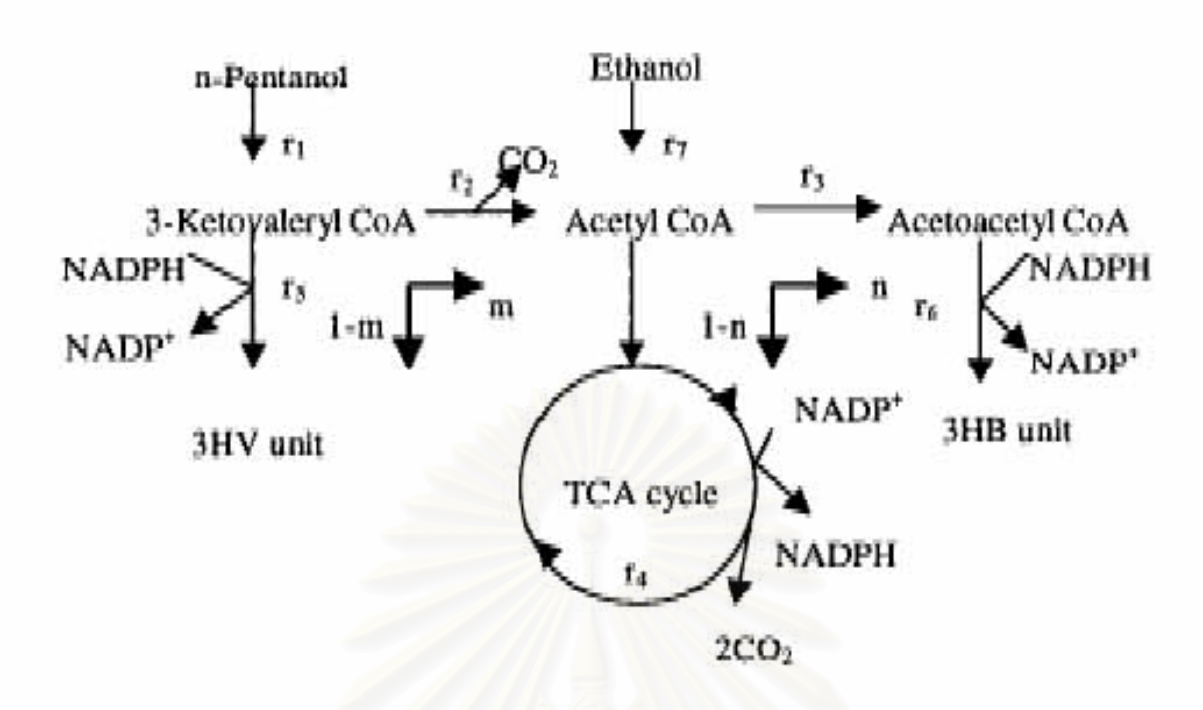


Figure 4.2-6: Metabolic networks for simple metabolic flux analysis of P(3HB) biosynthesis in *Paracoccus denitrificans* ATCC 17741 growing on a mixture of ethanol and n-pentanol as proposed by Chanprateep et al. (2001).

In 1997, Shi et al. proposed three sorts of metabolic networks portraying the P(3HB) biosynthesis in *R. eutropha* H16 growing heterotrophically on three types of organic acids as a sole carbon and energy source. These P(3HB)-yielding substrates were acetate, butyrate, and lactate. Although it is the different bacterial strain and also different carbonaceous substrate comparing to ours, this publication is worthwhile reviewing since it is the only one available conducting metabolic flux analysis of 'a whole picture' of PHA biosynthesis. In particular, it is frequently referred to in this research. Their proposed metabolic network is illustrated in *Figure 4.2-7*.

Obviously, these three networks shared most metabolic sub-networks; namely, simplified pattern of PP pathway, TCA cycle, ammonium assimilation through GDH system and residual biomass formation, and energy generation and reducing power regeneration. Still, there were such distinctions among them.

First, it was believed that each substrate was taken up through different systems. Acetate kinase and phosphate acetyltransferase is active when the microbe is growing on acetate. While β-oxidation plays a key role in butyrate uptaking and degrading system. Whilst lactate is taken up and directly converted to pyruvate.

Secondly, gateway to gluconeogenic pathway was somewhat different. In acetate- and butyrate-growing case, phosphoenolpyruvate (PEP) is synthesized from oxaloacetate (OAA) by the action of PEP carboxykinase. In addition, pyruvate (PYR) is attained from malate (MAL) via NADH-linked malic enzyme. On the other hand, PYR can be directly obtained from the up-taken lactate. And then PYR is converted to PEP by the action of PEP synthetase. This different depiction is interesting since it was implicated with one part of our work when deciding which route is present if *R. eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source. Allegedly, this will be covered in the next section.

Thirdly, 3-HB formation routes were also dissimilar. 3-HB monomeric unit can be obtained by condensation of two moles of acetyl coenzyme A (AcCoA) when the microbe is growing on acetate or lactate. Or it is directly produced without decomposition of the butyrate skeleton into AcCoA. The former route is totally the same as the one presenting in our work.

And the last distinction was at the anaplerotic pathway. Since PYR can be obtained directly from the up-taken lactate; as a result, OAA can be simply replenished from PYR by the action of PEP carboxykinase. In addition, there is no need for any other anaplerotic pathways. In contrast, glyoxylate shunt is operating as soon as acetate or butyrate is used as a carbon source. Again, these different anaplerotic pathways are of curiosity when constructing our metabolic networks of interest. Likewise, this will be covered in the next section.

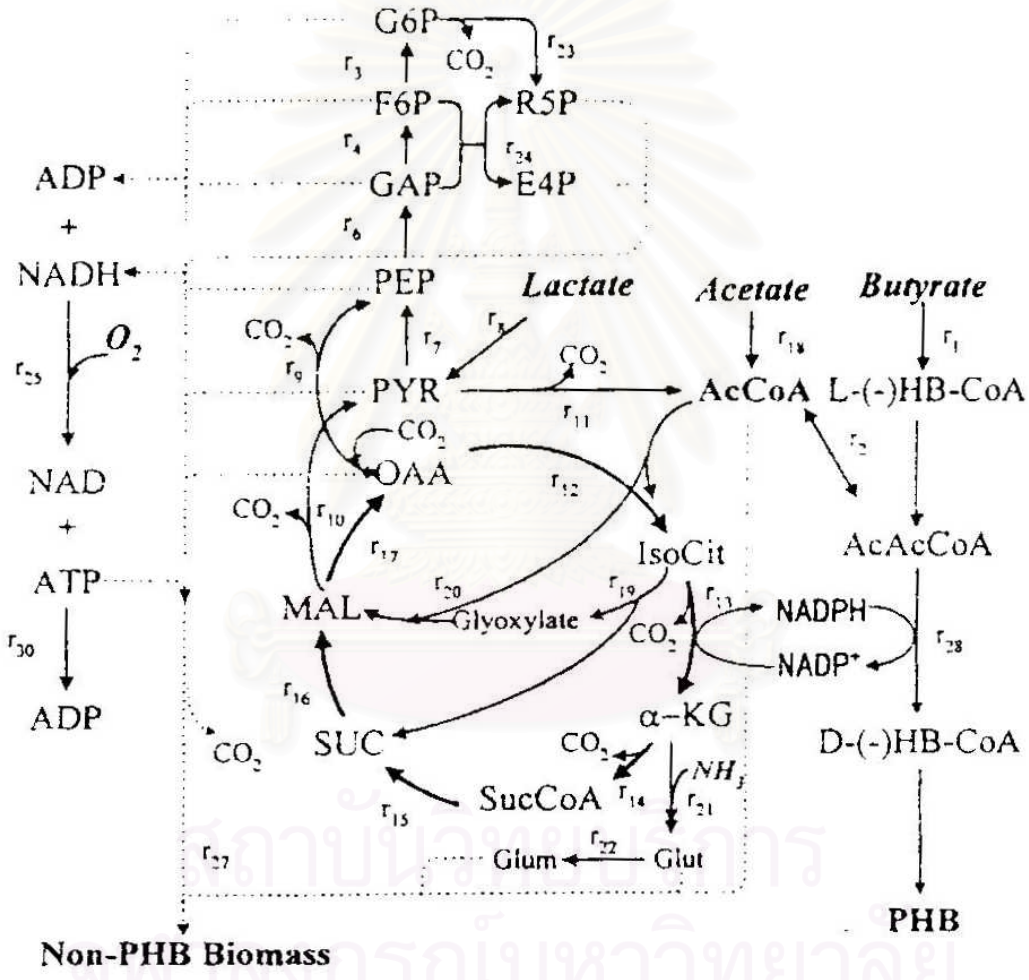


Figure 4.2-7: Metabolic networks for metabolic flux analysis of P(3HB) biosynthesis in *Ralstonia eutropha* H16 growing on various carbon sources as proposed by Shi et al. (1997).

Another attractive publication is that of Shi et al. (1999). Three cases of metabolic networks for P(3HB) biosynthesis in wild-type and recombinant *Escherichia coli* were constructed and analyzed as illustrated in *Figure 4.2-8*. The first one was used for describing metabolic behavior of a glucose-grown wild-type *E. coli*. The second was for a glucose-grown phosphoglucose isomerase-deleted (pgi^-) mutant. And the last for a gluconate metabolism in wild-type *E. coli*.

Each network was then analyzed by applying linear programming technique to yield flux distribution rendering the maximum P(3HB) biosynthesis. To achieve so, all other routes taking part in substrate conversion were totally omitted. Hence, there was no need to include nitrogen assimilation route and residual biomass formation into these three networks. Likewise, the fluxes representing the anaplerotic pathway and acetate secretion were both set to zero.

Concerning the resemblance of the proposed networks, they contained fully-oxidative PP pathway, TCA cycle, 3-HB formation route, and energy generation. While discrepancy was at central metabolism and also reducing power regeneration through transhydrogenase. For the first two cases, EMP pathway was present in the networks while linear mode of ED pathway was embraced in the third case.

Another intriguing issue is the presence of transhydrogenase. For normal growth of *E. coli* on both glucose and gluconate, transhydrogenase was presumed to be absent. Contradictorily, it was necessary to include the transhydrogenase effect in the mutant case; otherwise no feasible solution could be obtained. Unfortunately, they didn't discuss in physiological sense why transhydrogenase is merely present in the mutant case.

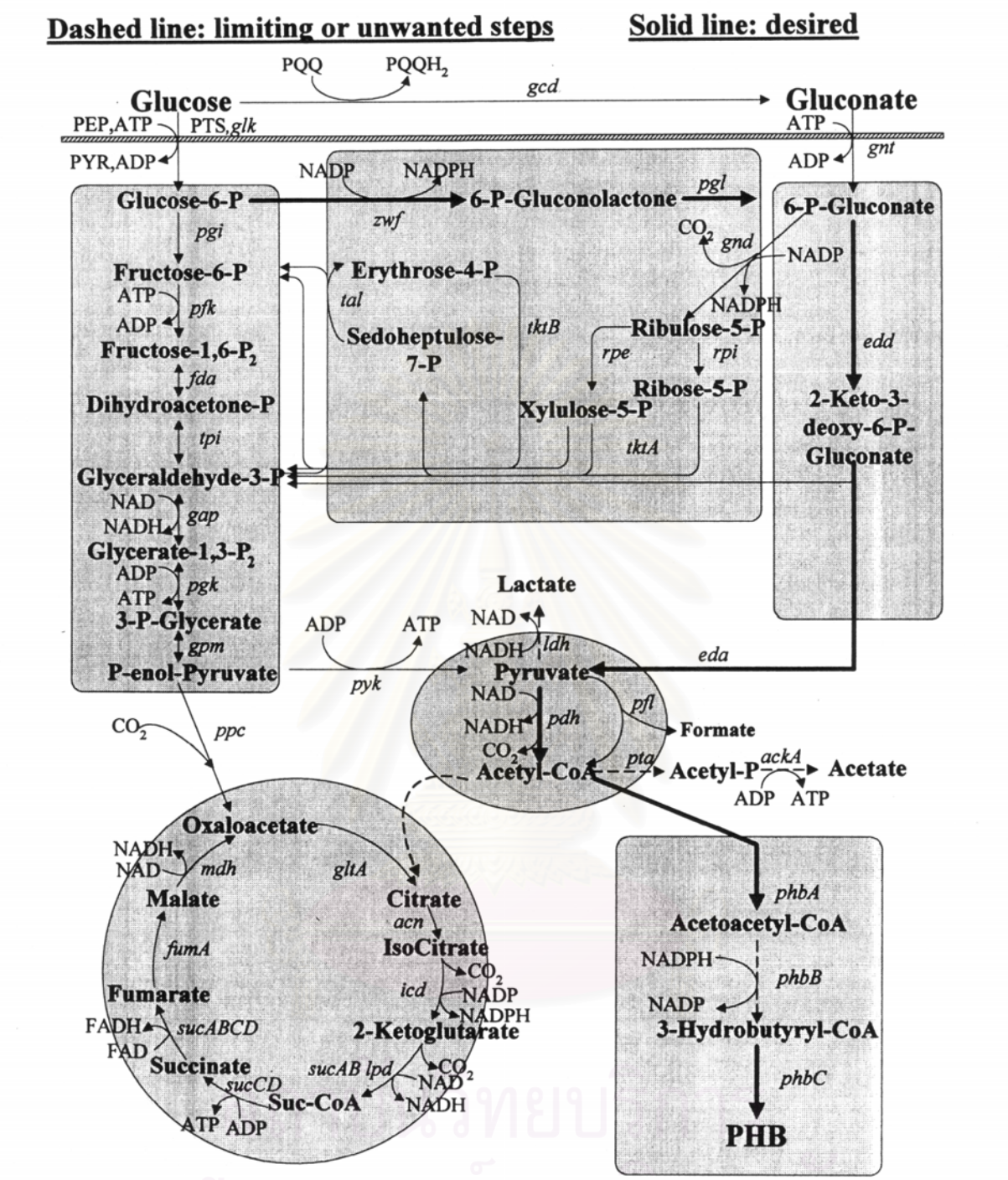


Figure 4.2-8: Metabolic networks for metabolic flux analysis of P(3HB) biosynthesis in *Escherichia coli* and recombinant *E. coli* growing on glucose or gluconate as a sole carbon and energy source as proposed by Shi et al. (1999).

In conclusion, there are three main approaches of study on development of a metabolic network.

The first one is principally based on the physiological, enzymic, and genomic evidence. It only indicates that which pathway is operating by the action of which enzyme encoded by which genomic operon. Specifically speaking, it could answer why but not when the pathway is present in the cell. And another drawback is that it rarely reveals any possible connections or entanglements of more than one pathway. Still, it is considered as the first evidence in constructing the model of interest. Thanks to its simple and straightforward task that tempts numerous works to focus on it. For instances, Schobert and Bowien (1984), and Br™mer and Steinb⎜chel (2002), and Horswill and Escalante-Semerena (1999).

The second concentrates more on connections of the pathways in the metabolic network. Simple combination of more than one pathway is primarily employed together with straightforward flux calculation. In general, it could elicit lots of thought-provoking explanations derived from miniature flux distribution. But crucial suggestions could not thoroughly be drawn out in regard with metabolic engineering sense. Selected publications of this approach are Wong et al. (1999), Yu and Wang (2001), and Chanprateep (2001).

Considering as the most powerful one, complete metabolic network map could suppress these mentioned pitfalls. It collects all possible reaction steps occurring in the cell, especially, energy generation and reducing power regeneration, which is not present in the first two approaches. Frequently, ‘a whole picture’ of cellular metabolism is tightly imposed on energy and reducing power constraints. Unfortunately, little effort on this approach has been achieved. Novel publications of Shi et al. [Shi et al., 1997; Shi et al., 1999] are following this approach.

Apparently, there is still a gap in the development of metabolic network that can depict the biosynthesis of either P(3HB) or P(3HB-*co*-3HV) in *R. eutropha* NCIMB 11599. Collecting pieces of clues from numerous studies, this thesis is defying proposing a metabolic network that is the most biochemically-feasible and mathematically-solvable.

4.3 Crucial hypotheses

To construct the metabolic networks of interest, inevitably, the following hypotheses are to be raised.

1. Due to little available knowledge on biochemistry of *Ralstonia eutropha* NCIMB 11599, the bacterial strain of interest, that of the neighboring strain of *R. eutropha* and even other bacterial genus is, in certain cases, applied in constructing the metabolic network of interest. This postulation has been employed by almost all cited references conducting a metabolic flux analysis.
2. It is rationally presumed that the bacterial strain of interest and even other bacterial genus stated in this section are not substantially pertaining in the evolution. As a result, the biochemistry information obtained so far is still well in service. For instances, supported by the review of Romano and Conway, carbohydrate metabolic pathways in certain bacteria has been slowly evolved. [Romano and Conway, 1996] Likewise, the conventional tricarboxylic acid (TCA) cycle needs no evolution since it is demonstrated to be the best possible pathway design according to an opportunism approach. [Mel⌈ndez-Hevia et al., 1996]
3. As already mentioned in *Chapter 2: LITERATURE REVIEW*, any fermentative pathways are not incorporated into any metabolic networks of interest. In addition, no excretion of any intermediary metabolites is putatively included. Seemingly, this assumption is applicable since the dissolved oxygen tension in the fermentation protocol was always kept so high above the oxygen-deficiency conditions stated in that of Vollbrecht and Schlegel. [Vollbrecht and Schlegel, 1978a, 1978b, and 1979; Vollbrecht et al., 1979; Schlegel and Vollbrecht, 1980]
4. All metabolic networks of interest are dealing with only heterotrophic metabolism. Mixotrophy, an existence of both heterotrophic and autotrophic capabilities, is not concerned. Since autotrophy, in general, contributes considerably less in cellular metabolism. [Personal communication with Bowien, 2003]

4.4 Metabolic networks of interest

For the sake of simplicity, the metabolic networks of interest can be categorized into three cases, depending upon the relevant carbonaceous substrate. The first one designated as case A is for describing the aerobic cultivation on glucose as a sole carbon and energy source. The second represented as case B is, in turn, for the aerobic cultivation on propionic acid as a sole carbon and energy source. Whilst the last, case C, is for the aerobic cultivation on a mixture of glucose and propionic acid.

Rationale behind the inclusion of any biochemical reactions is reviewed in this topic. In addition, for convenience, all key characteristics of those three cases can be consulted from *Table 4.4-1*. Details on stoichiometry of metabolic reactions can be consulted in Appendix B.

Table 4.4-1: Key characteristics of the metabolic networks of interest.

Characteristics	Network		
	A	B	C
Carbon source	Glucose	Propionic acid	Glucose/propionic acid
Included biochemical reactions	- Cyclic ED pathway (v_1-v_7) - Modified PP pathway (v_9-v_{10}) - AcCoA metabolism (v_{14}) - TCA cycle (v_{16}-v_{20}) - 3HB formation route (v_{21}-v_{22}) - Ammonium assimilation and residual biomass formation (v_{25}-v_{27}, v_{31}) - Energy generation and reducing power regeneration (v_{28}-v_{30}, v_{34}) - Respiration (v_{32}-v_{33}) - Anaplerotic pathway (v_{35}-v_{36})	- Gluconeogenic pathway (v_2, v_4-v_8) - Modified PP pathway (v_9-v_{10}) - MCA cycle (v_{11}-v_{13}) - AcCoA metabolism (v_{14}) - TCA cycle (v_{16}-v_{20}) - 3HB and 3HV formation routes (v_{21}-v_{24}) - Ammonium assimilation and residual biomass formation (v_{25}-v_{27}, v_{31}) - Energy generation and reducing power regeneration (v_{28}-v_{30}, v_{34}) - Respiration (v_{32}-v_{33}) - Anaplerotic pathway (v_{35}-v_{36})	- Cyclic ED pathway (v_1-v_7) - Modified PP pathway (v_9-v_{10}) - MCA cycle (v_{11}-v_{13}) - AcCoA metabolism (v_{14}) - TCA cycle (v_{16}-v_{20}) - 3HB and 3HV formation routes (v_{21}-v_{24}) - Ammonium assimilation and residual biomass formation (v_{25}-v_{27}, v_{31}) - Energy generation and reducing power regeneration (v_{28}-v_{30}, v_{34}) - Respiration (v_{32}-v_{33}) - Anaplerotic pathway (v_{35}-v_{36})

Table 4.4-1 (cont.): Key characteristics of the metabolic networks of interest.

Characteristics	Network		
	A	B	C
Carbon source	Glucose	Propionic acid	Glucose/propionic acid
Included key intermediary metabolites	G6P, F6P, GN6P, GA3P, G3P, PEP, PYR, R5P, E4P, AcCoA, OAA, IsoCit, AKG, SUC, MAL, AcAcCoA, GLUT, GLUM, ATP, NADH, NADPH, $FADH_2$, NH_3, CO_2, O_2	G6P, F6P, GN6P, GA3P, G3P, PEP, PYR, R5P, E4P, AcCoA, ProCoA, OAA, MetCit, IsoCit, AKG, SUC, MAL, AcAcCoA, KetVaCoA, GLUT, GLUM, ATP, NADH, NADPH, $FADH_2$, NH_3, CO_2, O_2	G6P, F6P, GN6P, GA3P, G3P, PEP, PYR, R5P, E4P, AcCoA, ProCoA, OAA, MetCit, IsoCit, AKG, SUC, MAL, AcAcCoA, KetVaCoA, GLUT, GLUM, ATP, NADH, NADPH, $FADH_2$, NH_3, CO_2, O_2
Number of fluxes	26^a (+3)	30^a (+3)	31^a (+3)
Number of key intermediary metabolites	25	28	28
Degree of freedom	1^b (+3)	2^b (+3)	3^b (+3)
Measured fluxes	- Specific glucose uptake rate (v_1) - Specific 3HB formation rate (v_{22}) - Specific growth rate (v_{27}) - Specific nitrogen uptake rate (v_{31})	- Specific propionic acid uptake rate (v_{11}) - Specific 3HB formation rate (v_{22}) - Specific 3HV formation rate (v_{24}) - Specific growth rate (v_{27}) - Specific nitrogen uptake rate (v_{31})	- Specific glucose uptake rate (v_1) - Specific propionic acid uptake rate (v_{11}) - Specific 3HB formation rate (v_{22}) - Specific 3HV formation rate (v_{24}) - Specific growth rate (v_{27}) - Specific nitrogen uptake rate (v_{31})
Number of measured fluxes	4	5	6
Model classification	Overdetermined by 3^c (0)	Overdetermined by 3^c (0)	Overdetermined by 3^c (0)

Note: Superscript ‘a’ indicates number of fluxes of the base-case network. The number in the parenthesis is the maximal number of fluxes that will be added to obtain all other cases.

Superscript ‘b’ indicates degree of freedom of the base-case network. The number in the parenthesis is the maximal degree of freedom that will be added to obtain all other cases.

Superscript ‘c’ indicates degree of redundancy of the base-case network. The number in the parenthesis is the minimal degree of redundancy of all other cases. 0 means the model is exactly-determined.

4.4.1 Metabolic network for the aerobic cultivation on glucose as a sole carbon and energy source

The metabolic network for the aerobic cultivation on glucose as a sole carbon and energy source comprises eight main sub-networks; namely, modified pattern of Entner-Doudoroff (ED) pathway, simplified pattern of pentose-phosphate (PP) pathway, acetyl coenzyme A (acCoA) formation, tricarboxylic acid (TCA) cycle, 3-HB formation route, glutamine synthetase-glutamate synthase (GS-GOGAT) pathway and residual biomass formation, energy generation and reducing power regeneration, and anaplerotic pathway. As the combination of those sub-networks, the metabolic network of interest is illustrated as *Figure 4.4-1*.

According to substantial studies, the operation of the cyclic mode ED pathway, represented as v_1-v_7, is an only one route of glucose catabolism in *R. eutropha*. However, suffering from mathematical difficulties associated with linear dependency, it calls for certain modification, say v_8 the interconversion of fructose-6-phosphate and glucose-6-phsophate is excluded. (See more details in topic 4.5.1: Linear dependency)

Modified pattern of PP pathway, labeled as v_9-v_{10}, is taken into account as it serves as an anabolic function for generation of two key precursor metabolites; ribulose-5-phosphate (R5P) and erythose-4-phosphate (E4P).

Acquired from the ED pathway, pyruvate (PYR) is further converted to acetyl coenzyme A (AcCoA) via the action of pyruvate dehydrogenase complex (PDC) in the lump reaction v_{14}.

After that AcCoA is partly oxidatively burnt in the TCA cycle yielding carbon dioxide (CO_2), energy in the form of ATP, NADH, and $FADH_2$, and, importantly, currency metabolite for biosyntheses NADPH. The TCA cycle is designated as v_{16}-v_{20}.

The rest of AcCoA channels to 3HB formation route, ciphered as v_{21}-v_{22}. As nitrogen limitation is achieved, this route favors the net synthesis of biopolymer reserve with negligible drive back from its degradation.

Ammonium is taken up, assimilated and anticipated in the formation of residual biomass, coded as v_{31}, v_{25}-v_{26}, and v_{27}, respectively. In spite of its small contribution, it still, inevitably, operate since there is evidence of cell growth even during the nitrogen limitation.

Besides substrate phosphorylation, generation of energy in the form of ATP can be fulfilled by oxidative phosphorylation, v_{28}-v_{29}. Due to other unaccounted ATP-linked process, excess dissipation of ATP is also present as v_{30}.

Another common metabolic activity is cellular respiration. Oxygen is inhaled and carbon dioxide is exhaled out of the cell, assigned as v_{33} and v_{32}, respectively. Although *R. eutropha* could assimilate and utilize carbon dioxide, this mixotrophic capability insignificantly contributes to the metabolic behavior. (As already mentioned in topic 4.3: Crucial hypotheses)

All stated metabolic pathways are assuredly participated in cellular metabolism of *R. eutropha*. We announce the contributions of all as base case metabolic network, simply called as A32. Still, there must be certain modifications by adding transhydrogenation and/or possible anaplerotic pathway by PEP carboxykinase (v_{35}) and/or additional source of NADPH production by NADPH-linked malic enzyme (v_{36}). As a result of all possible combinations, case A1-A31 can be sorted out. (See Appendix C for more details on the characteristics of all possible combinatorial metabolic network cases) Nevertheless, some are making sense in both biochemically-feasible and mathematically observable ways and some are not. To verify this, metabolic pathway analysis is allegedly carried out. (See topic 4.5: Metabolic pathway analysis for more details)

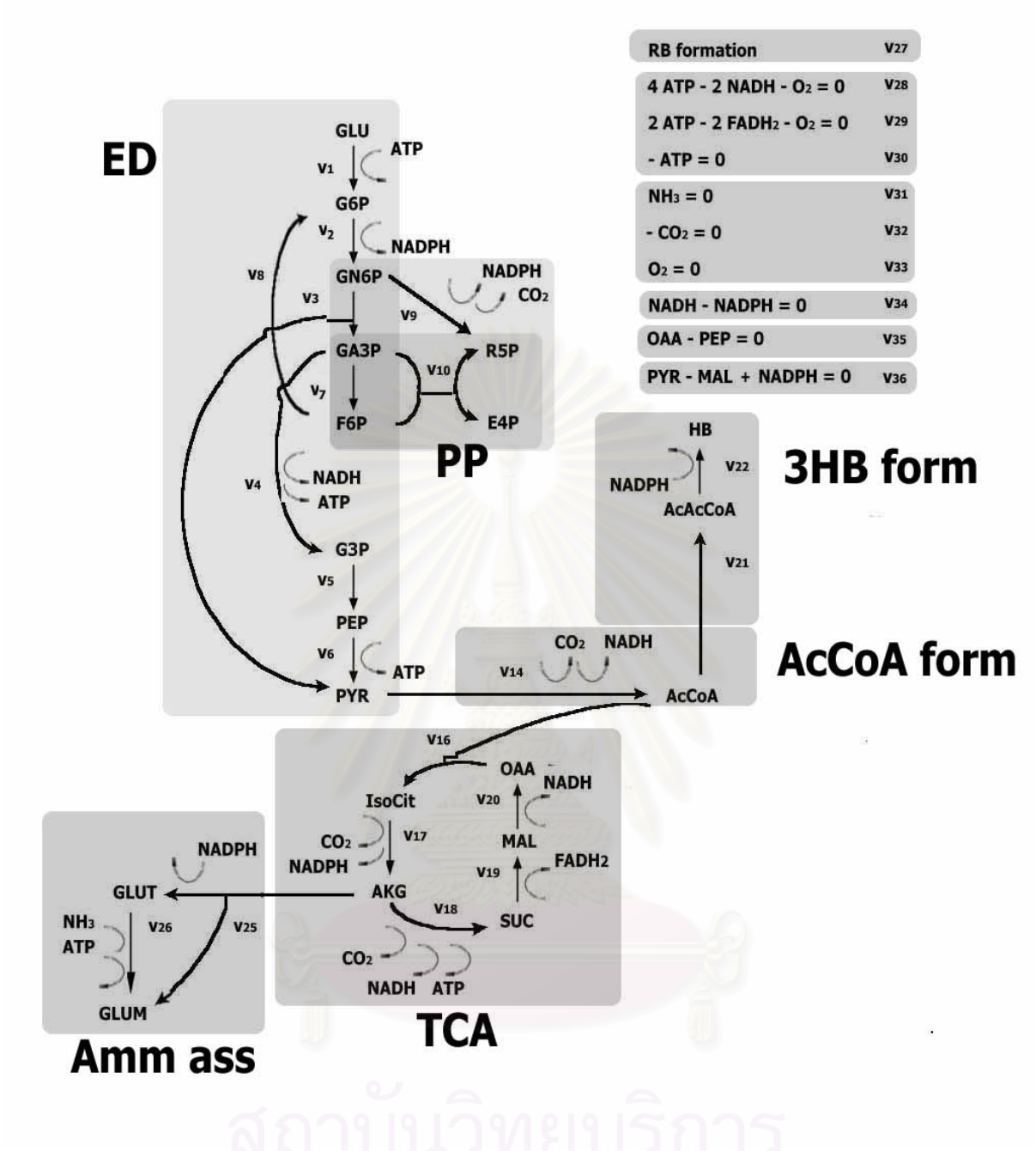


Figure 4.4-1: Proposed metabolic network case A describing the aerobic cultivation of *R. eutropha* NCIMB 11599 on glucose as a sole carbon and energy source.

4.3.2 Metabolic network for the aerobic cultivation on propionic acid as a sole carbon and energy source

The metabolic network for the aerobic cultivation on propionic acid as a sole carbon and energy source consisted of nine main sub-networks; namely, methylcitric acid (MCA) cycle, the gluconeogenesis pathway, simplified pattern of pentose-phosphate (PP) pathway, acetyl coenzyme A (acCoA) formation, tricarboxylic acid (TCA) cycle, 3-HB and 3-HV formation routes, glutamine synthetase-glutamate synthase (GS-GOGAT) pathway and residual biomass formation, energy generation and reducing power regeneration, and anaplerotic pathway. As the combination of those sub-networks, the metabolic network of interest is pictorially sketched as *Figure 4.4-2*.

According to substantial studies, the operation of the MCA cycle, represented as v_{11}-v_{13}, is dealing with propionic acid catabolism in *R. eutropha*.

To account on generation of triose and hexose, gluconeogenic pathway, represented as v_2, v_4-v_8, is to be mentioned. It can be noticed that gluconeogenic pathway resembles the reverse direction of the cyclic mode of Entner-Doudoroff (ED) pathway except for one point. The cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDPG) by the action of KDPG aldolase, v_3, though reversible, cannot service for gluconeogenic function.

Acquired from the MCA cycle, pyruvate (PYR) is further converted to acetyl coenzyme A (AcCoA) via the action of pyruvate dehydrogenase complex (PDC) in the lump reaction v_{14}. Decarboxylation of propionyl coenzyme A (ProCoA) to AcCoA, marked as v_{15}, is likely not a rational way to generate AcCoA in views of both biochemical and mathematical aspects. (See more details in topic 4.5.1: Linear dependency)

In addition to entering the TCA cycle and channeling to 3HB formation route, another remaining part of AcCoA can condense with the remaining ProCoA (the rest of ProCoA that is not catabolized via the MCA cycle) in 3HV formation route, ciphered as v_{23}-v_{24}. Likewise, as nitrogen limitation is achieved, this route favors the net synthesis of biopolymer reserve with negligible drive back from its degradation.

Simplified pattern of PP pathway (v_9-v_{10}), TCA cycle (v_{16}-v_{20}), 3HB formation route, (v_{21}-v_{22}), ammonium assimilation (v_{31} and v_{25}-v_{26}), residual biomass formation (v_{27}), oxidative phosphorylation (v_{28}-v_{29}), and cellular respiration (v_{32} and v_{33}) are exactly the same as already mentioned in the previous topic. As a result, they will not be covered in this topic again.

All stated metabolic pathways are assuredly participated in cellular metabolism of *R. eutropha*. We announce the contributions of all as base case metabolic network, simply called as B16. Still, there must be certain modifications by adding transhydrogenation (v_{34}) and/or possible anaplerotic pathway by PEP carboxykinase (v_{35}) and/or additional source of NADPH production by NADPH-linked malic enzyme (v_{36}). As a result of all possible combinations, case B1-B15 can be sorted out. (See Appendix C for more details on the characteristics of all possible combinatorial metabolic network cases) Nevertheless, some are making sense in both biochemically-feasible and mathematically observable ways and some are not. To verify this, metabolic pathway analysis is allegedly carried out. (See topic 4.5: Metabolic pathway analysis for more details)

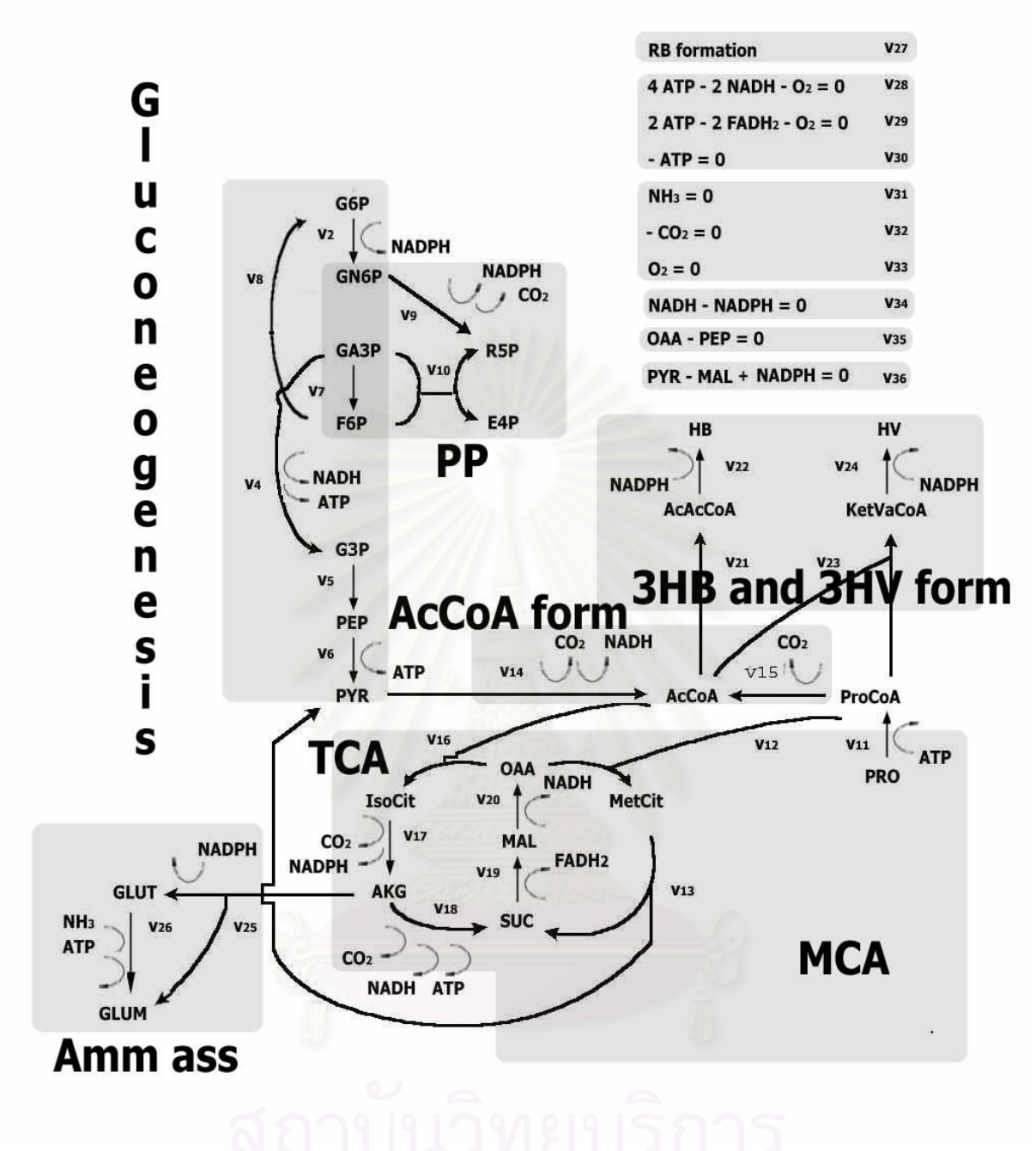


Figure 4.4-2: Proposed metabolic network case B describing the aerobic cultivation of *R. eutropha* NCIMB 11599 on propionic acid as a sole carbon and energy source. The estimated flux values of v4, v5, and v6 are to be negative to ensure their gluconeogenic function. Hence, their directions are opposite to the directions of the corresponding arrows.

4.3.3 Metabolic network for the aerobic cultivation on a mixture of glucose and propionic acid

The metabolic network for the aerobic cultivation on a mixture of glucose and propionic acid was composed of nine main sub-networks; namely, the modified pattern of Entner-Doudoroff (ED) pathway, simplified pattern of pentose-phosphate (PP) pathway, methylcitric acid (MCA) cycle, acetyl coenzyme A (AcCoA) formation, tricarboxylic acid (TCA) cycle, 3-HB and 3-HV formation routes, glutamine synthetase-glutamate synthase (GS-GOGAT) pathway and residual biomass formation, energy production and reducing power regeneration, and anaplerotic pathway. As the combination of those sub-networks, the metabolic network of interest is shown in *Figure 4.4-3*.

According to substantial studies, the operation of the cyclic mode ED pathway, represented as v_1-v_7, is an only one route of glucose catabolism in *R. eutropha*. However, suffering from mathematical difficulties associated with linear dependency, it calls for certain modification, say v_8 is excluded. (See more details in topic 4.5.1: Linear dependency)

According to considerable studies, the operation of the MCA cycle, represented as v_{11}-v_{13}, is dealing with propionic acid catabolism in *R. eutropha*.

Despite the presence of propionic acid, gluconeogenic pathway is not of primal concerned as in the metabolic network case B. Owing to the presence of glucose, glycolysis is induced and predominantly suppresses the gluconeogenic side. In this thesis, all fed-batch cultivations of *R. eutropha* growing on various compositions of a glucose-propionic acid mixture showed preponderant glucose uptake rate compared to that of propionic acid. Thus, it is lucid that in *R. eutropha* growing on a mixture of glucose and propionic acid glycolysis dominates over gluconeogenesis. This might be a result of cellular intelligence to avoid inhibitory effects from excessive uptake of a more toxic substrate, that is in this case propionic acid. In turns, we are curious of the controversial study of van Gulik and Heijnen (1995). They developed a metabolic network describing metabolism of *Saccharomyces cerevisiae* growing on a mixture of glucose and ethanol. Deliberately, they varied the uptake rate ratio of both substrates and speculate the corresponding flux distributions. Five different metabolic flux regimes were predicted upon transition from 100% glucose to 100% ethanol. Those five regimes can be simply encircled into three cases; namely, glycolysis-predominant, gluconeogenesis-predominant, and both equally-participated. Although intriguing recommendations can be drawn out from those simulation results, it is dubious whether those can be realized in experimentally aspects. As we suspect the cellular intelligence that might regulate the uptake of more toxic compound. Seemingly, this regulation tends to tune to glycolysis-prevailing.

Simplified pattern of PP pathway (v_9-v_{10}), TCA cycle (v_{16}-v_{20}), 3HB and 3HV formation routes, (v_{21}-v_{24}), ammonium assimilation (v_{31} and v_{25}-v_{26}), residual biomass formation (v_{27}), oxidative phosphorylation (v_{28}-v_{29}), and cellular respiration (v_{32} and v_{33}) are exactly the same as already mentioned in the previous topic. As a result, they will not be covered in this topic again.

All stated metabolic pathways are assuredly participated in cellular metabolism of *R. eutropha*. We announce the contributions of all as base case metabolic network, simply called as C64. Still, there must be certain modifications by adding transhydrogenation and/or possible anaplerotic pathway by PEP carboxykinase (v_{35}) and/or additional source of NADPH production by NADPH-linked malic enzyme (v_{36}). As a result of all possible combinations, case C1-C63 can be sorted out. (See Appendix C for more details on the characteristics of all possible combinatorial metabolic network cases) Nevertheless, some are making sense in both biochemically-feasible and mathematically observable ways and some

are not. To verify this, metabolic pathway analysis is allegedly carried out. (See topic 4.5: Metabolic pathway analysis for more details)

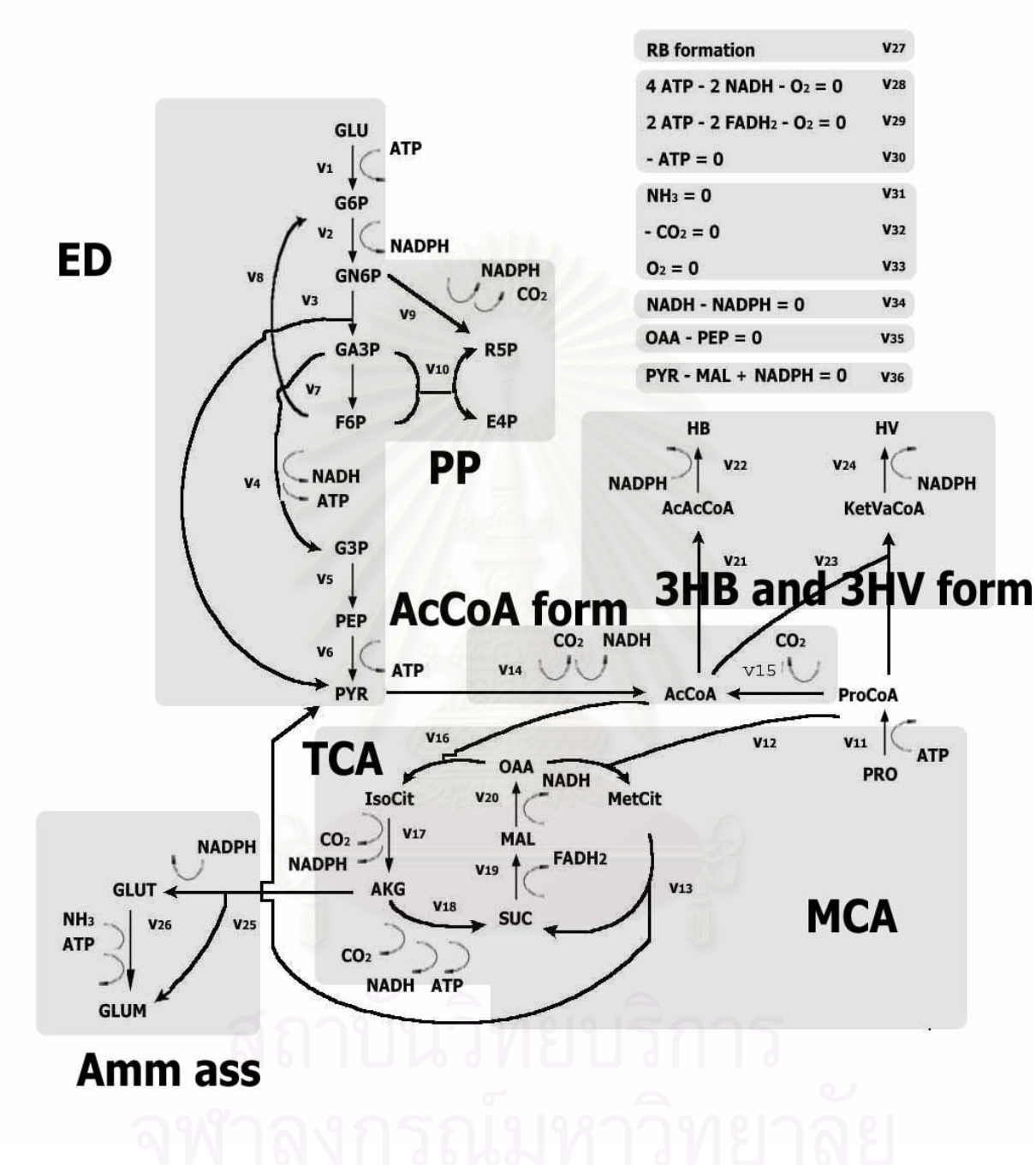


Figure 4.4-3: Proposed metabolic network case C describing the aerobic cultivation of *R. eutropha* NCIMB 11599 on a mixture of glucose and propionic acid.

4.5 Mathematically-feasible metabolic networks

After the collection of biochemical information, varieties of metabolic networks can be obtained as already mentioned in the former topic. Before performing flux calculation, these metabolic networks are to be analyzed mathematically. After that, the qualified metabolic network can be further subject to metabolic flux analysis as it is feasible in both biochemical and mathematical senses.

Technically, a mathematically-feasible metabolic network is the one that does not encounter any problems resulting from linear dependency. Moreover, its condition number should lie within the acceptable range. Details of which will be covered in this topic as follows.

4.5.1 Linear dependency

When handling of a linear algebraic system, the term 'linear dependency' is of primal concerned. Supposing that linear dependency occurs, the corresponding solution could be obtained but it is considered to be a trivial one.

Linear dependency can be observed once one row (or column) is a combination of others. This can be plainly checked by determining a rank of the system. Thanks to the availability of a number of competent software packages, the corresponding rank of a linear algebraic system can be easily obtained. In this thesis, we determine the rank of the pseudo-inverse matrix, $(G_c)^{\#}$, by the application of MATLAB m-file. The source code can be consulted in Appendix E. (See Chapter 3 for more details why the rank of $(G_c)^{\#}$ is to be checked.)

In particular, the following important points are to be noticed before performing flux calculations.

1. In metabolic network case A and C, where glucose is taken up and degraded via the cyclic mode of Entner-Doudoroff (ED) pathway, linear dependency could be observed as soon as v_7 and v_8, catalyzed by fructose-1,6-bisphosphatase (FBP) and phosphoglucose isomerase (PGI), respectively, are included altogether in most cases. (Except A6 and C14) Additionally, all other cases in which linear dependency is not troubling give irrational flux distributions. (See Chapter 5 for more details.) The mentioned exclusion of v_8 is still physiologically rational. Since glucose-6-phosphate (G6P) could be sufficiently provided from the up-taken glucose with no or little contribution of regeneration from fructose-6-phosphate (F6P). Then just sufficient amount of F6P is further channeled into the pentose phosphate (PP) pathway (represented as v_9-v_{10}) and residual biomass formation in the forward direction. It could be explained in the sense of metabolic flux analysis that the calculated flux values of v_2, v_3, and v_7 already represent the net forward direction, covering over the comparatively small reversible flux value of v_8. In contrast, linear dependency problem is totally vanishing in metabolic network case B.

2. In metabolic case B and C, where 3HV could be biosynthesized, linear dependency could be observed if v_{15} is included in all cases. This implies that flux analysis by metabolite-balancing technique cannot differentiate two routes that accomplishing acetyl coenzyme A (AcCoA) formation from propionyl coenzyme A (ProCoA). The one is direct decarboxylation of ProCoA to AcCoA represented as v15. And the other is simply considered as a combination effect of ProCoA metabolism through the

methylcitric acid (MCA) cycle (v_{12}-v_{13}) and conversion of pyruvate (PYR) to AcCoA by the action of pyruvate dehydrogenase complex (PDC) enzymes (v_{14}). The presence of v_{15} is proposed by the research group of Doi [Doi et al., 1986; Doi et al., 1987] but Buckel argued that it is not possible according to a chemical point of view. [Personal communication with Buckel, 2003] In this thesis, we support Buckel's idea and thus exclude v_{15} from our proposed metabolic networks.

3. Regarding the fact that AcCoA could not be directly obtained by decarboxylation of ProCoA, the operation of glyoxylate shunt is putatively not included as a possible anaplerotic pathway in the metabolic network case B. It is evident that *R. eutropha* channels PYR derived from propionic acid into the TCA cycle via AcCoA by the action of PDC enzymes. [Personal communication with Escalante-Semerena, 2002] Once PDC represented as v_{14} and glyoxylate shunt are bound together in the metabolic network, linear dependency can be inevitably observed. This is consistent with one comment in the textbook edited by Stephanopoulos et al. (1998). Also, it is known that the glyoxylate shunt is repressed by carbohydrate, including glucose. [White, 2000] Thus, in this thesis the glyoxylate shunt is putatively set aside in all metabolic network case A, B, and C.

4. In all metabolic case A, B, and C, linear dependency could be noticed as soon as two ammonium assimilation routes are coherently included. This observation was consistent with the suggestion in the textbook edited by Stephanopoulos et al. (1998). Considering the fact that ammonium concentrations in all fermentation protocol were kept so low that nitrogen limitation was achieved, the operation of (GS-GOGAT) pathway alone is practicable. The stated GS-GOGAT is realized as v_{25}-v_{26}.

5. Including transhydrogenation in the metabolic network might cause a trouble associated with linear dependency. (See Appendix C: Metabolic pathway analysis for those troublesome cases) However, it is not known yet whether transhydrogenation is present in *R. eutropha*. Furthermore, if it exists, there has been no conclusive report in which cultivation conditions it plays an important role in cellular metabolism. In this thesis, we try to answer these intriguing questions as will be covered in the next chapter.

6. As well, the exact anaplerotic pathway for regeneration of oxaloacetate in *R. eutropha* is not clearly suggested. This research could proudly suggest the most possible replenishing routes as will be covered in the next chapter.

4.5.2 Sensitivity analysis

The sensitivity analysis of metabolic network can be widely classified into two approaches. The first one is dealing with computation of condition number. While the other is accommodated through flux sensitiveness.

It can be simply seen that the first approach is the sensitivity analysis of a developed metabolic model. It reflects the sensitiveness of only the model itself. As a result, it can be determined before performing flux calculation.

In contrast, it is recommended to proceed the second approach before conducting an experiment. Because the obtained results could hint the experimenter to be aware of possible effects caused by inaccurate measurement.

4.5.2.1 Condition number

Before commencing flux calculations, condition number is to be first computed. This is required for solving a linear algebraic system. Apparently, the larger the condition number is, the more ill-conditioned the system.

Thanks to the well-known MATLAB built-in function, the condition numbers of all generated metabolic network cases could be comfortably obtained. Obviously, the case that is of full rank certainly yields the condition number that lies within the acceptable range – in the order of hundreds. (This criterion range can be consulted in chapter 3: Theoretical background) This indicates that all these cases yield reliable results when they are solved. The computed condition numbers of all cases that qualify for both biochemical and mathematical aspects are tabulated in *Table B4, B5, and B6* (in Appendix B: Metabolic pathway analysis) for metabolic network case A, B, and C, respectively.

4.5.2.2 Flux sensitivity

As for metabolic network case A, where *R. eutropha* NCIMB 11599 is growing on glucose as a sole carbon and energy source, metabolic network case A14 is selected to explain its metabolic behavior. (See Chapter 5: Metabolic flux analysis for more detail) Accordingly, the most influencing measurable rate is 3HB formation route, while ammonium uptake rate is the least. The order of superiority is as follows; 3HB formation rate > residual biomass formation rate > glucose uptake rate > ammonium uptake rate. Hence, measurement of 3HB formation rate in FB 07, where glucose is used as a sole carbon and energy source, should be carefully conducted as its error might influence the calculated results considerably.

Regarding metabolic network case B, where *R. eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source, metabolic network case B13 is selected to describe its metabolic behavior. (See Chapter 5: Metabolic flux analysis for more detail) Correspondingly, the most influencing measurable rate is 3HV formation rate, while residual biomass formation rate is the least. The order of superiority is as follows; 3HV formation rate > 3HB formation rate > propionic acid uptake rate > ammonium uptake rate > residual biomass formation rate. Hence, measurement of 3HV formation rate in FB 08, where propionic acid is used as a sole carbon and energy source, should be carefully conducted as its error might influence the calculated results considerably.

Concerning metabolic network case C, where *R. eutropha* NCIMB 11599 is growing on a mixture of glucose and propionic acid, two metabolic network cases are selected to elucidate its metabolic behavior; case C29 and C30. (See Chapter 5: Metabolic flux analysis for more detail)

As for metabolic network case C29, the most influencing measurable rate is glucose uptake rate, while residual biomass formation rate is the least. The order of superiority is as follows; glucose uptake rate > 3HV formation rate > 3HB formation rate > propionic acid uptake rate > ammonium uptake rate > residual biomass formation rate.

Considering metabolic network case C30, the most influencing measurable rate is 3HB formation rate, while ammonium uptake rate is the least. The order of superiority is as follows; 3HB formation rate > residual biomass formation rate > 3HV formation rate > glucose uptake rate > propionic acid uptake rate > ammonium uptake rate.

When focusing on the individual flux, v_{30}, representing ATP maintenance reaction, is of the largest value for all mentioned metabolic network cases. This implies that this flux is the most sensitive comparing to the others when performing flux calculations.

Details on each flux sensitivity of all mentioned cases are tabulated in Appendix C: Metabolic pathway analysis.

CHAPTER 5
METABOLIC FLUX ANALYSIS

5.1 Flux calculation
5.2 Identification of metabolic pathways in proposed metabolic networks
5.3 Estimation of non-measured rates and reconciliation of measured rates
5.4 Determination of maximum theoretical molar fraction of 3HV monomeric unit in copolymer
5.5 Identification of principal nodes and possible bottlenecks in proposed metabolic networks
5.6 Suggestion of rational modifications for metabolic engineering

Following the achievement of development of metabolic network in the previous chapter, now it is the time to perform metabolic flux analysis, which is assuredly our objective of this research. As such, this chapter will cover all results and discussion of metabolic flux analysis. Accordingly, there are six sections in this chapter.

In the first section, we start with basic flux calculation. At the end of section 5.1, flux distributions of all fermentation protocols are preparatory in hand for further analysis in the subsequent section.

Then, identification of metabolic pathways in metabolic networks is carried out in section 5.2. Crucially, this section serves as the first objective of this research.

As a bonus, non-measured rates are successfully estimated as well as measured rates are rationally reconciled. This will be covered in section 5.3.

Next, maximum theoretical molar fraction of 3-hydroxyvalerate (3HV) monomeric unit in copolymer is figured out in section 5.4. This section can be realized as the second objective of this research and its conclusion could lead to the next section.

Subsequently, section 5.5 will cover details on identification of principal nodes and possible bottlenecks in metabolic networks. Indeed, this section embodies the third objective of this research.

Last but not least, the fourth objective is the fact that we could suggest such a rational modification for metabolic engineering to debottleneck the ones proposed in the previous section. Finally, we end this chapter by suggesting rational modifications for metabolic engineering in section 5.6.

5.1 Flux calculation

As already developed in the previous chapter, all metabolic network cases that qualify for both biochemical and mathematical constraints are prompt to proceed into the next stage. Specifically, flux calculation is to be successively performed.

To perform flux calculation, all possible measured rates are first to be figured out. This can be done by taking a mathematical model into a time-series concentration profile. [See Chapter 3: Theoretical background for more details] Details and examples on how to evaluate all measured and evaluated rates of all fermentation protocols in this research are covered in Appendix A.

Back to the topic, technically, there are two approaches to perform flux calculation. The first one is the exact solution. It is based on the assumptions that there is no noise in both measurement errors and pseudo-steady state approximation. Estimated fluxes of this approach can be solved according to *Equation 3.3-7.* The second is the least-square solution where the mentioned assumptions are relaxed. This method can be solved according to *Equation 3.3-11*. In fact, both approaches are applied in this research, but it appears that the latter approach yields more reliable results as will be explained as follows.

As an example, estimated fluxes for fermentation protocol of FB01 are selected on purpose. Flux distributions estimated from both approaches are illustrated in *Table 5.1-1*. As can be seen, in each approach there are further four sub-columns. These sub-columns denoted as C14, C26, C29, and C30 are referred to the qualified cases discussed in chapter 4 and that will be proceeded in the next section for identification of the most suitable case. It is noteworthy to state that in this section we will only expose differences occurring between the two approaches. As a result, we will not discuss any differences occurring between metabolic network cases.

To determine which approach is more reliable, it is wise to inspect at their results. Speaking of which, to do so it is needed to perceive such ideas in the succeeding section of identification of metabolic pathways in metabolic networks. Specifically, the results turn to be irrational if there is an inconsistency in enzymic information. (Details will be elucidated in the next section)

By inspecting the results in *Table 5.1-1*, it can be seen that there are negative values of v_{23} for all metabolic network cases if we assume that there is no noise in measurement errors and pseudo-steady state approximation. In contrast, if we allow such a noise, there is a positive value of v_{23} in metabolic network case C30. Since v_{23} represents the net formation of 3HV, it is urged that its physiological function should be in the direction of formation of 3HV. Hence, v_{23} should be positive. (See the next topic for the sign of v_{23} according to its physiological function) Accordingly, it can be concluded that the results obtained by the first approach are less rational than those obtained by the second approach. This implies that there exists such a noise in either measurement errors or pseudo-steady state approximation in this fermentation protocol.

Not only this fermentation protocol, but also all other protocols that the least-square solution proves to be more rational than the exact solution. The least-square method could turn the signs of the estimated fluxes to be consistent with their enzymic information in several cases. Hence, it is wise to take the noise into account when further performing flux calculation for all fermentation protocols.

To support this idea, Goel *et al*. (1993) also suggested the least-square method be applied in flux calculation instead of the exact one. They found a negative value of an irreversible flux using the exact solution, but not in the least-square solution. Thus, they used the least-square solution for further analysis as it was believed to be more reliable. In addition, Stephanopoulos *et al*. (1998) recommended it as a more favorite in various studies on metabolic flux analysis.

After the appropriate approach is selected, flux distributions of all qualified metabolic network cases for all fermentation protocols can be obtained. This can be done by solving the linear algebraic system of interest. Thankfully, we do that comfortably by the application of MATLAB m-file. (See Appendix E for a detailed source code m-file) Collectively, all computed results are tabulated in Appendix D.

One intriguing remark is to be pointed out. To compare all flux distributions in metabolic flux analysis, estimated fluxes need to be normalized on the same basis. Conventional studies has simply used uptake rate of a substrate as a normalizer. Hence, a value in a flux distribution is reported as the occurring of that flux if 100 moles of a substrate are consumed. But for the case of a mixture of various substrates, total mole carbon consumed is widely used as a normalizer. [Goel *et al*., 1995; Wahlbom, 2001] Consequently, this research will use this as a normalizer for all cases. Specifically, the normalizer of interest is as follows.

First, the uptake rates of glucose and propionic acid, defined as $v_{1,unnorm,mol\ basis}$ and $v_{11,unnorm,mol\ basis}$, respectively, are converted into C-mol basis. This can be easily done according to *Equation 5.1-1*.

$$v_{i,unnorm,C-mol\ basis} = \frac{v_{i,unnorm,mol\ basis}}{(6v_{1,unnorm,mol\ basis} + 3v_{11,unnorm,mol\ basis})} \quad ; \quad i = 1,11 \tag{5.1-1}$$

Then the unnormalized term undergoes the normalization by the following expression. It can be noticed that after the rendition the summation of the normalized terms of both glucose and propionic acid uptake rates is equal to 100 as desired.

$$v_{i,norm,C-mol\ basis} = v_{i,unnorm,C-mol\ basis}\left(\frac{100}{(v_{1,unnorm,C-mol\ basis} + v_{11,unnorm,C-mol\ basis})}\right) \quad ; \quad i = 1,11 \tag{5.1-2}$$

Once the normalized terms of both glucose and propionic acid uptake rates are obtained, they will be used further as a normalizer for all other fluxes according to *Equation 5.1-3*.

$$v_{i,norm,C-mol\ basis} = \frac{v_{i,unnorm,mol\ basis}}{(v_{1,norm,C-mol\ basis} + v_{11,norm,C-mol\ basis})} \quad ; \quad i = 1-36\ except\ \ 1,11 \tag{5.1-3}$$

Table 5.1-1: Estimated fluxes of two approaches in flux calculation; with and without a noise in measurement errors and pseudo-steady state approximation. The flux calculation is performed with data of FB01, representing the fed-batch fermentation protocol in which the molar C/N ratio and %P were successively controlled at 32.7 and 14.7, respectively. All flux values are given in mol / total mol carbon consumed.

Flux	Without noise in measurement errors and pseudo-steady state approximation				With noise in measurement errors and pseudo-steady state approximation			
	C14	C26	C29	C30	C14	C26	C29	C30
v1	0.8652	0.8652	0.8652	0.8652	0.8652	0.8652	0.8652	0.8727
v11	0.1348	0.1348	0.1348	0.1348	0.1348	0.1348	0.1348	0.1273
v22	0.1272	0.1272	0.1272	0.1272	0.1272	0.1272	0.1272	0.1845
v24	0.0193	0.0193	0.0193	0.0193	0.0193	0.0193	0.0193	0.0696
v27	0.6049	0.6049	0.6049	0.6049	0.5594	0.5594	0.5594	0.6210
v31	0.2559	0.2559	0.2559	0.2559	0.2898	0.2898	0.2898	0.3259
v2	-0.2011	0.8525	0.8525	0.7731	-0.2401	0.8534	0.8534	0.8107
v3	-0.2338	0.8199	0.8199	0.7140	-0.2703	0.8232	0.8232	0.7609
v4	1.8353	0.7817	0.7817	0.5170	1.8816	0.7880	0.7880	0.6237
v5	1.7446	0.6909	0.6909	0.3998	1.7977	0.7041	0.7041	0.5142
v6	1.6795	0.6259	1.6795	0.0700	1.7109	0.6173	1.7109	0.2526
v7	-1.0493	0.0042	0.0042	0.0572	-1.0896	0.0039	0.0039	0.0371
v8	-1.0536				-1.0936			
v9	0.0327	0.0327	0.0327	0.0062	0.0302	0.0302	0.0302	0.0172
v10	0.0218	0.0218	0.0218	0.0483	0.0202	0.0202	0.0202	0.0387
v12	0.2060	0.2060	0.2060	0.1901	0.1992	0.1992	0.1992	0.1408
v13	0.2060	0.2060	0.2060	0.1371	0.1992	0.1992	0.1992	0.1081
v14	1.4805	1.4805	1.4805	0.7235	1.4815	1.4815	1.4815	0.9295
v16	1.1615	1.1615	1.1615	0.3463	1.1659	1.1659	1.1659	0.4085
v17	1.1615	1.1615	1.1615	0.2933	1.1659	1.1659	1.1659	0.3759
v18	1.2362	1.2362	1.2362	0.3150	1.2083	1.2083	1.2083	0.3882
v19	1.4422	1.4422	1.4422	0.4257	1.4075	1.4075	1.4075	0.4800
v20	1.4422	1.4422	2.4958	0.3992	1.4075	1.4075	2.5011	0.4637
v21	0.0819	0.0819	0.0819	0.1031	0.0854	0.0854	0.0854	0.1512
v23	-0.0260	-0.0260	-0.0260	-0.0048	-0.0225	-0.0225	-0.0225	0.0361
v25	0.3797	0.3797	0.3797	0.4061	0.3777	0.3777	0.3777	0.4376
v26	0.3022	0.3022	0.3022	0.3287	0.3238	0.3238	0.3238	0.3793
v28	3.0915	3.0915	3.0915	1.0717	3.0767	3.0767	3.0767	1.2995
v29	0.7211	0.7211	0.7211	0.2128	0.7038	0.7038	0.7038	0.2400
v30	14.7709	12.6638	13.7173	1.7996	14.8925	12.7053	13.7989	3.0111
v32	4.0353	4.0353	4.0353	1.2240	3.9742	3.9742	3.9742	1.6598
v33	3.8126	3.8126	3.8126	1.2846	3.7805	3.7805	3.7805	1.5395
v34		1.0536				1.0936		
v35	0.0336	0.0336	-1.0200	0.2718	0.0577	0.0577	-1.0359	0.2131
v36			-1.0536				-1.0936	

Note: Highlighted is the flux that violates its enzymic information.

5.2 Identification of metabolic pathways in proposed metabolic networks

To fulfill the first objective of this research, identification of metabolic pathways in various metabolic networks is to be carried out.

5.2.1 Rationale

It is believed that certain metabolic pathways are only active in metabolic network under certain conditions. In addition, some are widely reported to be present in tons of microorganisms, but some are not in certain microorganisms. Or even in the same microorganism, some are active in such conditions but surprisingly inactive in some conditions. These pathways could be any parts of central metabolism, anaplerotic pathways, or any special metabolic pathways for production of secondary metabolites.

Due to diversity of central metabolism schemes, various metabolic networks of a microorganism can be perceived. *Escherichia coli* is one of the most versatile microorganisms that can utilize carbohydrate via three glycolytic pathways. These three pathways are Embden-Meyerhof-Parnas (EMP), pentose-phosphate (PP), and Entner-Doudoroff (ED) pathways. However, all three might not operate at their fully-activated level at the same time. If glucose is used as a sole carbon and energy source, EMP and PP pathways are activated but ED is not. While ED and PP serve as the main glycolysis for gluconate metabolism. Additionally, between two present pathways, EMP serves at higher priority than PP. But the level of priority can be changed by certain operating conditions. [Nielsen, 1997]

In 1979, Aiba and Matsuoka performed metabolic flux analysis of citric acid fermentation by *Candida lipolytica*. As a base case, they constructed a simplified metabolic network consisting of EMP pathway, TCA cycle, glyoxylate shunt, pyruvate carboxylation, and formation of the major macromolecular pools, i.e., proteins, carbohydrates, and lipids. Their metabolic network can be consulted from *Figure 5.2-1*.

Obviously, their metabolic model was underdetermined since the degree of freedom was seven but only six reaction rates were measured. To solve the model, they imposed a constraint by setting one of the rates in the network equal to zero, and thus, the model turned to be an exactly-determined system. Three different cases were examined, reflecting three different models of citric acid biochemistry. In the first case model, the glyoxylate shunt is inactive (the flux to glyoxylate shunt is set to zero). As for the second case model, pyruvate carboxylase is inactive (this flux is zero). And in the last case model, TCA cycle is incomplete.

When performing flux calculations, it was found that some of the fluxes in the latter two cases were negative and thus violated their directionality of irreversibility. While there was no doubt with estimated flux values of the first model. Also, the *in vitro* measurement of the corresponding enzymes could support the flux calculation results.

The two anaplerotic routes, glyoxylate shunt and pyruvate carboxylation, obviously are necessary to replenish TCA cycle intermediates when citrate and isocitrate are secreted to the extracellular medium. However, when they successfully performed metabolic flux analysis, they concluded that glyoxylate shunt is inactive or operates at a very low rate in *C. lipolytica* under citric acid production conditions.

This study is worthwhile reviewing since it is the first one that propose the rational method how to select the most suitable metabolic network from all other possibility. As a matter of fact, it has inspired the following works on metabolic flux analysis aiming at identifying metabolic pathways in metabolic networks.

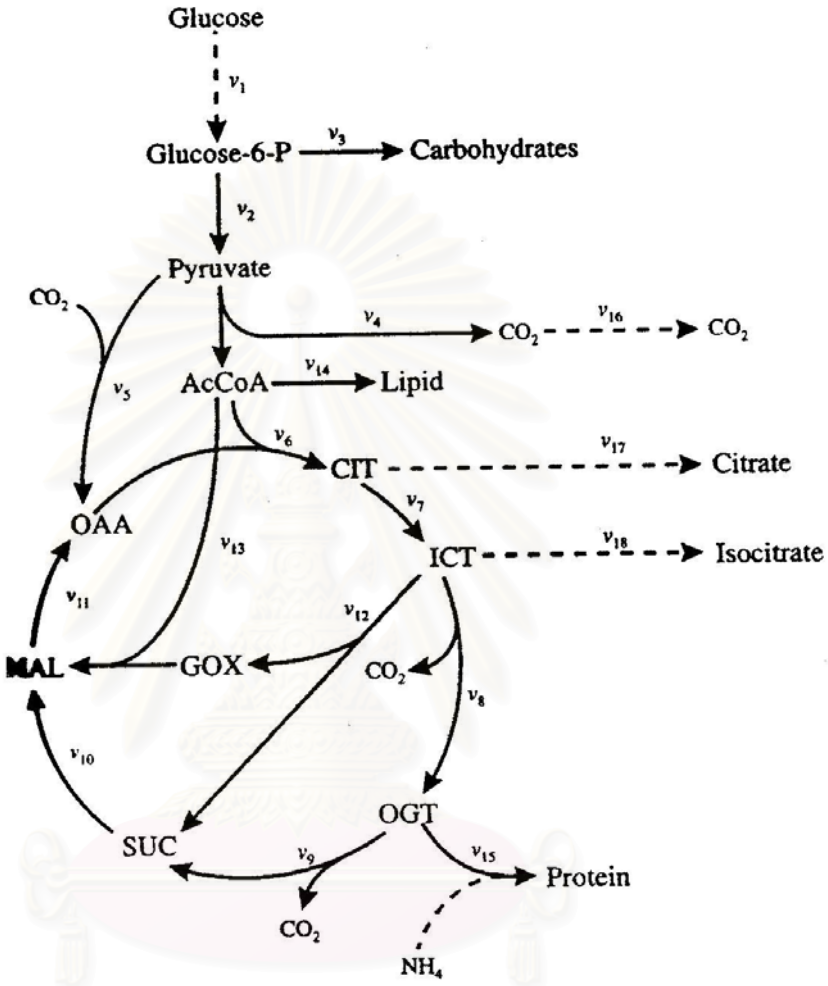


Figure 5.2-1: Metabolic network for *Candida lipolytica* proposed by Aiba and Matsuoka (1979). Abbreviations: G6P, glucose-6-phosphate; AcCoA, acetyl-CoA; OAA, oxaloacetate; CIT, citrate; ICT, isocitrate; OGT, α-ketoglutarate; SUC, succinate; MAL, malate; GOX, glyoxylate. Broken lines represent transport reactions, whereas solid lines represent intracellular reactions.

Another interesting publication is the study of Park *et al.* (1997). Accordingly, they constructed three mutants from the wild type *Corynebacterium glutamicum* to elucidate the presence of pyruvate kinase, two anaplerotic pathways and transhydrogenation. The mutants were phosphoenolpyruvate carboxylase-deficient mutant (designated as ppc mutant), pyruvate kinase-deficient mutant (designated as pyk mutant), and mutant that lacked both phosphoenolpyruvate carboxylase and pyruvate kinase (designated as ppc-pyk mutant). As such, they developed sixteen metabolic network cases corresponding to all possible permutations of the four key enzymes; namely, phosphoenolpyruvate carboxylase (PPC), pyruvate carboxylase (PC), pyruvate kinase (PYK), and transhydrogenase (THD).

Before performing flux calculations, they rejected some of these all metabolic network cases after thorough consideration. The qualified cases were those that are consistent with both biochemical and mathematical constraints.

Biochemical constraints were directly related to enzymic information. The biochemically-infeasible metabolic network case was the one that possess impossible flux. For examples, for pyk mutant, metabolic network cases that contain the flux of PYK were apparently biochemical-infeasible and thus rejected. Another example is the missing of all anaplerotic pathways. Metabolic network cases that excluded both PPC and PC were rejected for this matter.

As for mathematical constraints, they investigated the stoichiometric matrix of each metabolic network case. The metabolic case was mathematically-infeasible at the moment that its stoichiometric matrix is singular.

After passing the first screening procedure, the qualified cases were then underwent flux calculations. Only one most suitable case was then successfully sought out after examining the obtained flux distributions of all metabolic network cases. In this second stage of screening, metabolic network case was rejected only if there was any inconsistency in the estimated flux values. The inconsistency was imposed on directionality of reversibility and physiological sense. The example of the former category was negative flux values in TCA cycle. While negative value of the anaplerotic PPC flux fell into the latter category.

Apparently, their study is worthwhile reviewing since their procedure for the identification of the metabolic pathways in the metabolic networks is systematic and rational. Furthermore, it is much more in details than the study of Aiba and Matsuoka (1979). As such, we rely heavily on their procedures for the identification of the metabolic pathways in our metabolic networks. The first stage of culling is already covered in the previous chapter. This chapter will then go through the second stage.

This research will identify the four pathways whether they are active or not in various conditions. These four pathways are as follows.

- Cyclic mode of Entner-Doudoroff (ED) pathway is fully operative; v_7 and v_8 are simultaneous present.
- Transhydrogenation (v_{34}) is active as an additional source of NADPH production.
- Phosphoenolpyruvate carboxykinase (v_{35}) is the only route of anaplerotic pathway.
- NADPH-linked malic enzyme (v_{36}) serves itself as an additional source of NADPH production not as an anaplerotic pathway.

These four pathways were carefully selected for the study since it was found they are incorporated in only some cases under studied. While other pathways are always active under any conditions.

For a particular fermentation protocol, only one most suitable is to be rationally picked up among all possible metabolic network cases. This section will identify the most suitable pathway, which is the most thermodynamically-feasible, for all protocols and also summarize the rationale why it is selected whereas the others are rejected.

5.2.2 Criteria for pathway identification

Before commencing the identification of a metabolic pathway in a metabolic network, it is recommended to set up such a criteria. Traditionally, information on participating enzymes in the metabolic network is used as criteria. Summarized in *Table 5.2-1* are the criteria applied in this research. (Representative metabolic reaction of each flux can be consulted in Appendix B)

First, it is recommended to see whether the flux of interest is irreversible. This can be simply done by looking at the second main column. If the symbol '+' is indicated, this means the flux of interest is irreversible. Moreover, the estimated flux value is to be positive to fit with its directionality of irreversibility. (The term 'directionality of irreversibility is named according to Bonarius *et al*. (1997) who first defined it as the demand that a (number of) flux(es) is non-negative)

On the other hand, if no symbol is indicated, this means the flux of interest is truly reversible. Therefore, its sign can be either positive or negative. Specifically, its sign is the indicator of its physiological function. Correspondingly, description of the physiological function is also given in the third main column. As can be seen, the column is splitted into two sub-columns. These two sub-columns narrate the physiological function of the flux in case the flux is positive and negative, respectively. As such, if the flux is irreversible, the column is leaving blank.

For example, from *Table 5.1-1*, in metabolic network case C26, C29, and C30, it can be seen that the estimated v_2 is positive. (See Appendix C for more details on included reactions in each metabolic network case) But the estimated v_2 is negative in metabolic network case C14. According to the enzymic information of v_2, its sign is to be positive to fit with its directionality of irreversibility. Thus, it can be concluded that metabolic network case C14 yields an irrational result and it should be first rejected since there are better alternative cases where rational results can be obtained.

Furthermore, similar conclusion could be drawn out when looking at v_3. The estimated v_3 in metabolic network case C26, C29, and C30 is positive, while it is negative in metabolic network case C14. Although v_3 is reversible, its physiological function in metabolic network case C (where a mixture of glucose and propionic acid is used as a carbon and energy source) should be in the direction of glycolysis not gluconeogenesis. Hence, metabolic network case C14 is rejected by this matter.

Table 5.2-1: Information on participating enzymes in the metabolic network.

Flux	Directionality of irreversible reaction	Physiological function	
		+	-
v2	+		
v3		For glycolysis	For gluconeogenesis
v4		For glycolysis	For gluconeogenesis
v5		For glycolysis	For gluconeogenesis
v6		For glycolysis	For gluconeogenesis
v7	+		
v8		For formation of glucose-6-phosphate	For formation of fructose-6-phosphate
v9	+		
v10	+		
v12	+		
v13	+		
v14	+		
v16	+		
v17	+		
v18	+		
v19	+		
v20	+		
v21		For 3HB formation	For 3HB degradation
v23		For 3HV formation	For 3HV degradation
v25	+		
v26	+		
v28	+		
v29	+		
v30	+		
v32	+		
v33	+		
v34		For NADH production	For NADPH production
v35		Anaplerotic function	Gluconeogenic function
v36		Gluconeogenic function and NADPH production	Anaplerotic function

Note: + in the column of directionality of irreversible reaction indicates the flux is to be positive. Leaving blank means the flux is reversible and thus can be either positive or negative.

In the column of physiological function, brief explanation is stated according to its direction of flux, either + or -.

5.2.3 Thermodynamically-feasible metabolic network in *Ralstonia eutropha* when growing on glucose as a sole carbon and energy source

In FB07, Kasipar (2002) conducted a fed-batch fermentation protocol in which the molar C/N ratio and %P were successively controlled at 102 and 0, respectively. Obviously, it is associated with the metabolic network case A since glucose is used as a sole carbon and energy source. (See Appendix C for more details on included reactions in each metabolic network case)

From the previous stage of development of metabolic networks, there are seven metabolic network cases left that qualify for both biochemical and mathematical constraints; namely, metabolic network case A6, A10, A13, A14, A18, A21, and A22. (See details of each case in Appendix C) Among them, only one most suitable metabolic network case is to be picked up for describing the metabolic behavior of *R. eutropha* NCIMB 11599 of interest. This can be done by thorough inspecting the estimated fluxes of all cases. The following are the rationale for selecting the most suitable case. Details on flux distributions of all those stated cases can be consulted in *Table D7* in Appendix D.

First, metabolic network case A6 is rejected since it yields a negative flux value for v_7. Apparently, v_7 represents the irreversible conversion of glyceraldehyde-3-phosphate (GA3P) to fructose-6-phosphate (F6P). Hence, the negative value is contradictory to the enzymic information. (See *Table 5.2-1* for the corresponding enzymic information)

Unfortunately, metabolic network case A6 embodies the metabolic behavior where the cyclic mode of Entner-Doudoroff (ED) pathway fully operates. As a result, the most probable metabolic network of interest, in sense of metabolic flux analysis, turns to be dealing with a modified pattern of ED pathway – including only either v_7 or v_8.

Metabolic networks case A10, A13, and A14 are the cases where v_7 is included but v_8 is not. On the other hand, metabolic networks case A18, A21, and A22 are the cases where v_8 is included but v_7 is not. In the latter three metabolic networks, it can be noticed that the flux values of v_8, which represents the interconversion of glucose-6-phosphate (G6P) and F6P, are negative. Although this is consistent with its physiological sense, those networks are still rejected. (See *Table 5.2-1* for the corresponding enzymic information) Instead, metabolic networks case A10, A13, and A14 win over for this matter.

As already mentioned in the previous chapter, the absence of v_8 is still physiologically rational. Since G6P could be sufficiently provided from the up-taken glucose through v_2 with no or little contribution of regeneration from fructose-6-phosphate (F6P) through v_8. Then just sufficient amount of F6P is further channeled into the pentose phosphate (PP) pathway and residual biomass formation in the forward direction. It could be explained in the sense of metabolic flux analysis that the calculated flux values of v_2, v_3, and v_7 already represent the net forward direction, covering over the comparatively small reversible flux value of v_8.

One intriguing observation can be drawn out. The flux distribution of metabolic network case A10 is quite resemble to that of its equivalent case A18. (All reactions are similar except v_7 and v_8 – case A10 includes only v_7 but case A18 includes only v_8) The differences of each individual flux are clearly insignificant, ca. less than 5%. The above can also be seen with the other equivalent pairs – metabolic networks case A13 and A21, accompanied by case A14 and A22. This comfortably relaxes us when deciding to pick up only one most suitable metabolic network for further analysis.

Now among three remaining cases, metabolic network case A14 is finally selected. Because there is no strong contradiction on any flux values.

Specifically speaking, metabolic network case A10 is rejected since v_{34} is somewhat inconsistent with its physiological sense. (See *Table 5.2-1* for the corresponding enzymic information) Although transhydrogenation v_{34}, catalyzing the interconversion of NADPH and NADH, is reversible, its physiological role should be in the formation of NADPH from NADH. [Jones *et al*., 1975 reviewed in Joo, 1998]

While metabolic network case A13 is also rejected due to the fact that it yields a negative value for v_{35}. Considering as the anaplerotic pathway, v_{35} needs to be a positive value to make sure the oxaloacetate (OAA) replenishment from phosphoenolpyruvate (PEP) as it is named as PEP carboxykinase. (See *Table 5.2-1* for the corresponding enzymic information)

In conclusion, metabolic network case A14 is likely to be the most suitable one that can describe the metabolic behavior of the cell growing on the condition of fed-batch protocol of FB07 as shown as *Figure 5.2-2*. Three remarkable points are concomitantly drawn out. First, the cyclic mode of ED pathway are probably not fully operative. Secondly, transhydrogenase activity seems to be absent under this condition. And thirdly, PEP carboxykinase appears to be the only possible anaplerotic pathway when the cell is cultivated under this condition. Furthermore, all estimated fluxes of metabolic network case A14 for this protocol are summarized in *Table 5.2-2*.

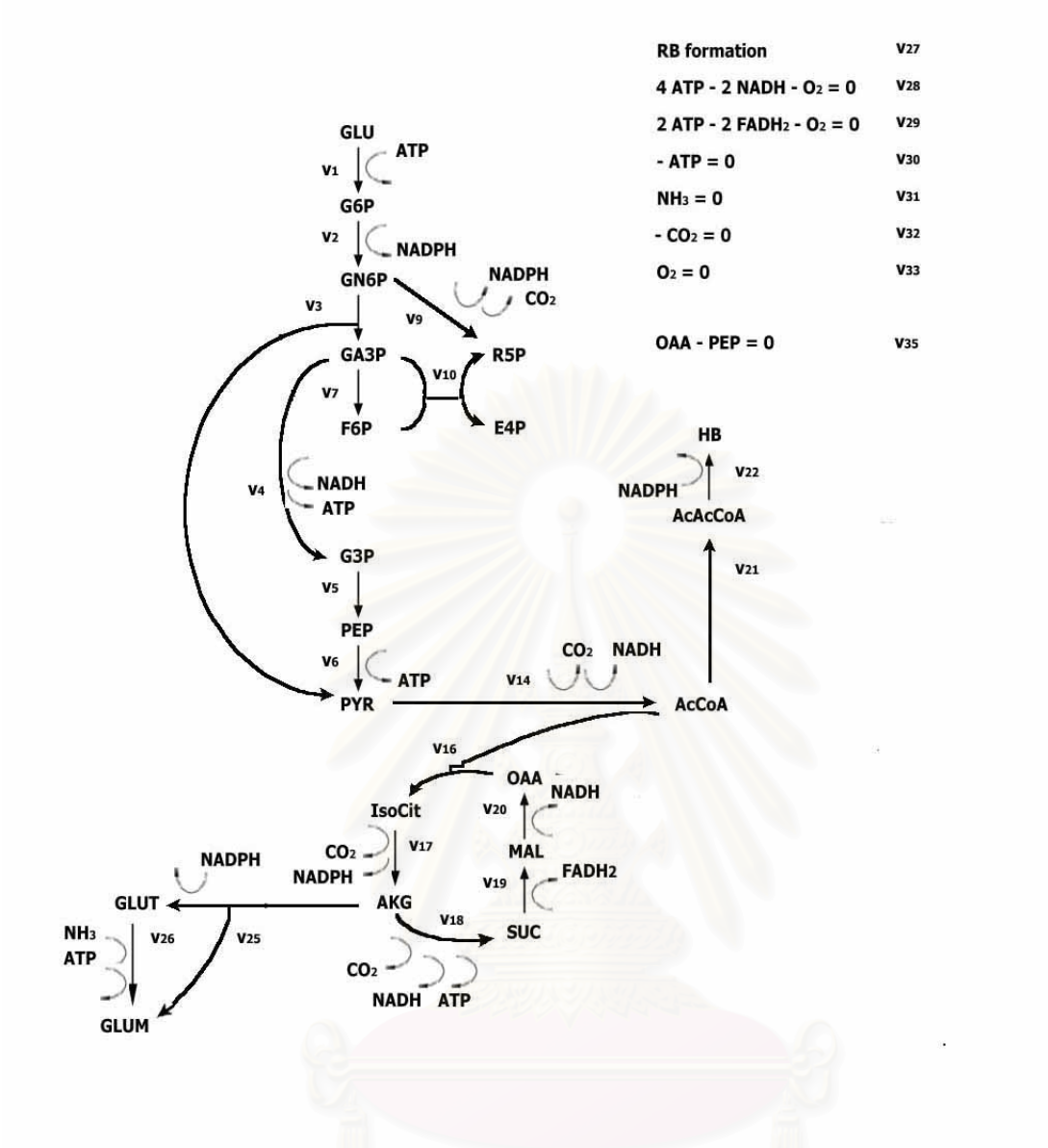


Figure 5.2-2: Proposed thermodynamically-feasible metabolic network when *Ralstonia eutropha* NCIMB 11599 is growing on glucose as a sole carbon and energy source.

5.2.4 Thermodynamically-feasible metabolic network in *Ralstonia eutropha* when growing on propionic acid as a sole carbon and energy source

In FB08, Kasipar (2002) conducted the fed-batch fermentation protocol in which the molar C/N ratio and %P were successively controlled at 102 and 100, respectively. Obviously, it is associated with the metabolic network case B since propionic acid is used as a sole carbon and energy source. As such, all evaluated measured rates of this fermentation protocol are applied to identify metabolic pathways in metabolic network case B.

From the previous stage of development of metabolic networks, there are three metabolic network cases left that qualify for both biochemical and mathematical constraints; namely, metabolic network case B10, B13, and B14. (See details of each case in Appendix C) Likewise, among them, only one metabolic network case is to be picked up for describing the

metabolic behavior of *R. eutropha* NCIMB 11599 of interest. This can be done by thoroughly examining the estimated fluxes of all cases. The following are the rationale for selecting the most suitable case. Details on flux distributions of all those stated cases can be consulted in *Table D8* in Appendix D.

Before moving on to the selection, one interesting point is to be mentioned. For all metabolic network cases, inclusion of both v_7 and v_8 does not turn the model problematic due to linear dependency. This indicates that the operation of the ED pathway should be fully active.

Going back to the selection, first, metabolic network case B14 is rejected since it yields negative flux values for v_7, v_{10}, and v_{35}, and also positive flux values for v_4 and v_6. (See *Table 5.2-1* for the corresponding enzymic information)

As already discussed in the previous topic, the negative values of v_7 and v_{35} are not acceptable. In addition, v_{10} needs to be a positive value to ensure the formation of a tetrose (erythrose-4-phosphate: E4P) and a pentose (ribose-5-phosphate: R5P) from a triose (glyceraldehyde-3-phosphate: GA3P) and a hexose (fructose-6-phosphate: F6P) by the action of transaldolase and transketolase. Hence, the negative value of v_{10} is likewise not agreeable.

As for v_4 and v_6, their physiological role is to be in the gluconeogenic fashion. Hence, v4 and v6 should be negative to ensure the conversion of 3-phosphoglycerate (G3P) to GA3P and pyruvate (PYR) to phosphoenolpyruvate (PEP), respectively.

Next, it can be easily seen that the remaining two candidates – case B10 and B13 – yield flux distributions that look somewhat alike. This implies that both are qualified over all constraints, especially NADPH requirement. The significant differences can be found for the estimated fluxes of v_6, v_{20}, and v_{35}. Apparently, these fluxes are located at the questionable node of PYR, PEP, and malate (MAL). To select only one case from these two cases, it is noteworthy to elucidate the carbon fluxes of both for the sake of comparison.

As a means of comparison, first we look at the malate node. If an NADPH-linked malic enzyme, representing as v_{36}, is active to fulfill NADPH production (case B13), the flux value of v_{20} (the conversion of malate to oxaloacetate) will be lower (when comparing to case B10 where v_{36} is not active) since the incoming flux of v_{19} (the formation of malate from succinate) is the same. And in order to maintain the constant flux value of the outgoing v_{12} and v_{16}, the flux value of v_{35} needs to be higher to compensate for the loss of v_{20}. Finally, the magnitude of v_6 should, accordingly, be higher as it acquires more carbon flux through the active v_{36}. Additionally, due to the higher magnitude of v_6, consumption of ATP associated with this flux is consequently higher. (the gluconeogenic v_6 is an ATP-consuming reaction.) This discrepancy allows a small different flux estimate of maintenance energy v_{30}.

But if v_{36} is inactive, v34 is responsible for NADPH production instead (case B10). Unfortunately, there is no report, so far investigated, that suggests the presence of transhydrogenase in any living cells growing on propionic acid as a sole carbon and energy source. Speaking of which, we suspect for its presence and thus presumably reject metabolic network case B10 owing to the fact that there is no report to veto our assumption. Nonetheless, future work should aim on confirming this aspect experimentally.

Moreover, according to the study of Schobert and Bowien (1984), the presence of an NADPH-linked malic enzyme is proved in *R. eutropha* heterotrophically growing on various carbon sources including pyruvate. (See details and discussion of this study in Chapter 2: Literature review.) Since propionic acid is oxidized to pyruvate by the action of the enzymes in methylcitric acid (MCA) cycle, this can connect, in such matter, those clues together.

In conclusion, metabolic network case B13 is likely to be the most suitable one that can describe the metabolic behavior of the cell growing on the condition of fed-batch protocol of FB08 as shown as *Figure 5.2-3*. Four remarkable points are concomitantly drawn out. First, the cyclic mode of ED pathway should fully operate as a gluconeogenic function.

Secondly, transhydrogenase activity is presumably absent under this condition. Thirdly, PEP carboxykinase appears to be the only possible anaplerotic pathway when the cell is cultivated under this condition. And fourthly, NADPH-linked malic enzyme is likely to be active in a gluconeogenic fashion for the sake of production of the additional NADPH. Furthermore, all estimated fluxes of metabolic network case B13 for this protocol are summarized in *Table 5.2-2*.

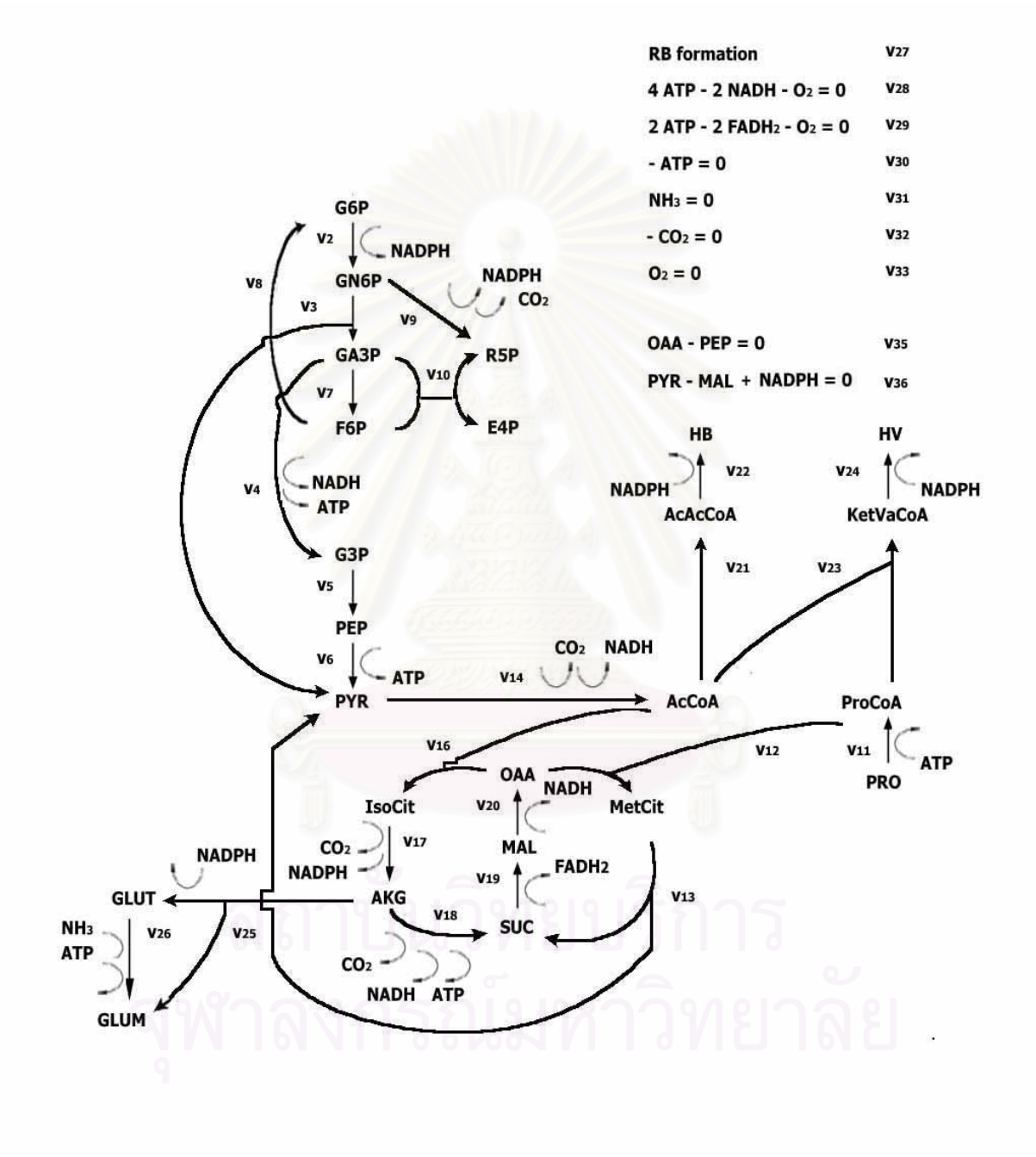


Figure 5.2-3: Proposed thermodynamically-feasible metabolic network when *Ralstonia eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source. The estimated flux values of v4, v5, and v6 are to be negative to ensure their gluconeogenic function. Hence, their directions are opposite to the directions of the corresponding arrows.

5.2.5 Thermodynamically-feasible metabolic network in *Ralstonia eutropha* when growing on a mixture of glucose and propionic acid

Concerning metabolic network case C, all the rest of fed-batch fermentation protocols fall into this category. Those protocols are FB01, FB02, FB03, FB04, FB05, and FB06 in which the molar C/N ratio and %P were successively controlled at 32.7 and 14.7; 171.3 and 14.7; 32.7 and 85.4; 171.3 and 85.4; 4 and 50; and 200 and 50, respectively. Also, the metabolic network case C includes the center-point runs designated as FB09, FB10, FB11, FB12, and FB13 in which the molar C/N ratio and %P were successively controlled at 102 and 50. As such, all evaluated measured rates of these fermentation protocols are applied to identify metabolic pathways in metabolic network case C.

From the previous stage of development of metabolic networks, there are four metabolic network cases left that are qualified for both biochemical and mathematical constraints; namely, metabolic network case C14, C26, C29, and C30. (See details of each case in Appendix C) Among them, only one metabolic network case is to be chosen for describing the metabolic behavior of *R. eutropha* NCIMB 11599 of interest. This can be done by thorough examining the estimated fluxes of all cases. The following are the rationale for selecting the most suitable case. Details on flux distributions of all those stated cases can be consulted in *Table D1-D6* and *D9-D13* in Appendix D.

It can be clearly noticed that metabolic network case C30 is the most probable one for all protocols except for FB06. Instead, FB06 is likely to concede to metabolic network case C29. The rationale for selection of the two metabolic cases will be covered as follows. For the sake of convenience, it will be covered distinctly as two groups; the one for only FB06 and the other for the rest.

First, we look at the flux distributions of all protocols except that of FB06. Metabolic network case C14 is rejected. This is evidenced by the fact that the estimated flux values of v_2 (the conversion of glucose-6-phosphate to 6-phosphogluconate) and v_3 (the cleavage of 6-phosphogluconate to glyceraldehyde-3-phosphate and pyruvate) are negative for all protocols in this group. In addition, it also yields the negative values of v_7 and v_8 for FB01, FB02, FB03, FB04, FB09, and FB12. (See *Table 5.2-1* for the corresponding enzymic information)

According to their enzymic information, v_2 and v_7 are irreversible. Thus, the estimated fluxes are to be positive. As for v_3 and v_8, although they are reversible, their physiological role should be in the glycolytic function. Hence, it should be positive likewise.

Next, metabolic network case C26 is rejected since it yields the positive value of v_{34}. Supporting idea is exactly the same as in the previous topic.

Then metabolic network case C29 is also rejected as the negative value of v_{35} can be observed. (See *Table 5.2-1* for the corresponding enzymic information) Likewise, supporting idea is exactly the same as in the previous topic. However, it can be marked that although the flux value of v_{35} for FB05 is not negative, metabolic network case C29 is still rejected. This is due to the fact that there is an inconsistency in v_{36}. The estimated flux of v_{36} for FB05 is unfortunately negative. This violates its physiological role as we previously mentioned. (See *Table 5.2-1* for the corresponding enzymic information)

Finally, metabolic network case C30 is the most suitable for FB01, FB02, FB03, FB04, FB05, FB09, FB10, FB11, FB12, and FB13. It can be observed that there is a small negative value of v_{23} for FB01, FB03, FB05, and FB13. This flux, catalyzing the condensation of acetyl coenzyme A with propionyl coenzyme A to form ketovaleryl coenzyme A, cannot be negative. (See *Table 5.2-1* for the corresponding enzymic information) This might be because v_{24}, the formation rate of 3HV monomeric unit, is so

small. It can be consulted from sensitivity analysis of the impact of 3HV formation rate on the estimated flux of v_{23}. Apparently, 3HV formation rate is the most influential comparing to the other measured rates. (See details in *Table C10* in Appendix C.) Only slight increase in 3HV formation rate could turn the estimated flux of v_{23} to be positive. This implies that the negative flux is resulted from either measurement error or model inconsistency. But for further metabolic flux analysis, we still use the flux distributions where v_{23} is negative to avoid accusation from distorted artifact.

As for protocol FB06, metabolic network case C30 is, on the other hand, unlikely to be selected. Because there is a negative value for v_{35}, which catalyzes the reaction between oxaloacetate (OAA) and phosphoenolpyruvate (PEP). This severely violates its physiological role since v_{35} is an anaplerotic pathway – replenishing OAA from PEP.

Considering the fact that metabolic network case C30 is rejected, one intriguing issue can be realized. It appears that NADPH production from conventional v_2, v_9, and v_{17} is likely insufficient. (All are catalyzed by NADPH-linked enzymes) An additional source for NADPH production is demanded to avoid the violation of pseudo-steady state approximation of NADPH. As such, metabolic network C14, C26, and C29 can serve more NADPH production.

Unluckily, there is no strong evidence to reject all other remaining cases on the basis of either physiological or enzymic inconsistencies. Before deciding which one is the most suitable, it is wise that thorough investigation of these three candidates is carried out first.

The difference among these three cases is at an additional source for NADPH production. Since this condition is the most severe case where nitrogen limitation is the utmost, an additional source for NADPH production is inevitably needed. The additional source for NADPH in metabolic network case C14 can be achieved by the high flux of v_2 resulted from the recycled v_8. (v_2 is an NADPH-linked reaction.) While transhydrogenation and NADPH-linked malic enzyme serve that role in metabolic network case C26 and C29, respectively.

As evidenced in all other protocols where a mixture of glucose and propionic acid is used for the cultivation, the cyclic mode of ED pathway does not fully operate and transhydrogenase is not present. It might be skeptical if only one protocol could possess the exceptionally distinct properties – having either full mode of cyclic ED pathway or transhydrogenase activity. But as for NADPH-linked malic enzyme, there is a clear evidence that this enzyme activity was found when *R. eutropha* is growing on several carbohydrates and intermediates of tricarboxylic acid (TCA) cycle. [Schobert and Bowien, 1984] As a result, presumably, metabolic network case C29 is ultimately selected.

In conclusion, metabolic network case C29 and C30, as illustrated as *Figure 5.2-4* and *Figure 5.2-5*, respectively, are likely to be the most suitable ones that can describe the metabolic behavior of the cell growing under the condition of fed-batch protocol of FB06 and all the rest, respectively. Four remarkable points are concomitantly drawn out. First, the cyclic mode of ED pathway cannot be fully operative. Secondly, transhydrogenase activity is presumably absent at all conditions. Thirdly, PEP carboxykinase appears to be the only possible anaplerotic pathway when the cell is cultivated on all conditions. And fourthly, NADPH-linked malic enzyme is likely to be active in a gluconeogenic fashion for the sake of production of the additional NADPH for FB06, where insufficient production of NADPH is claimed. Furthermore, all estimated fluxes for theses protocols are summarized in *Table 5.2-2*.

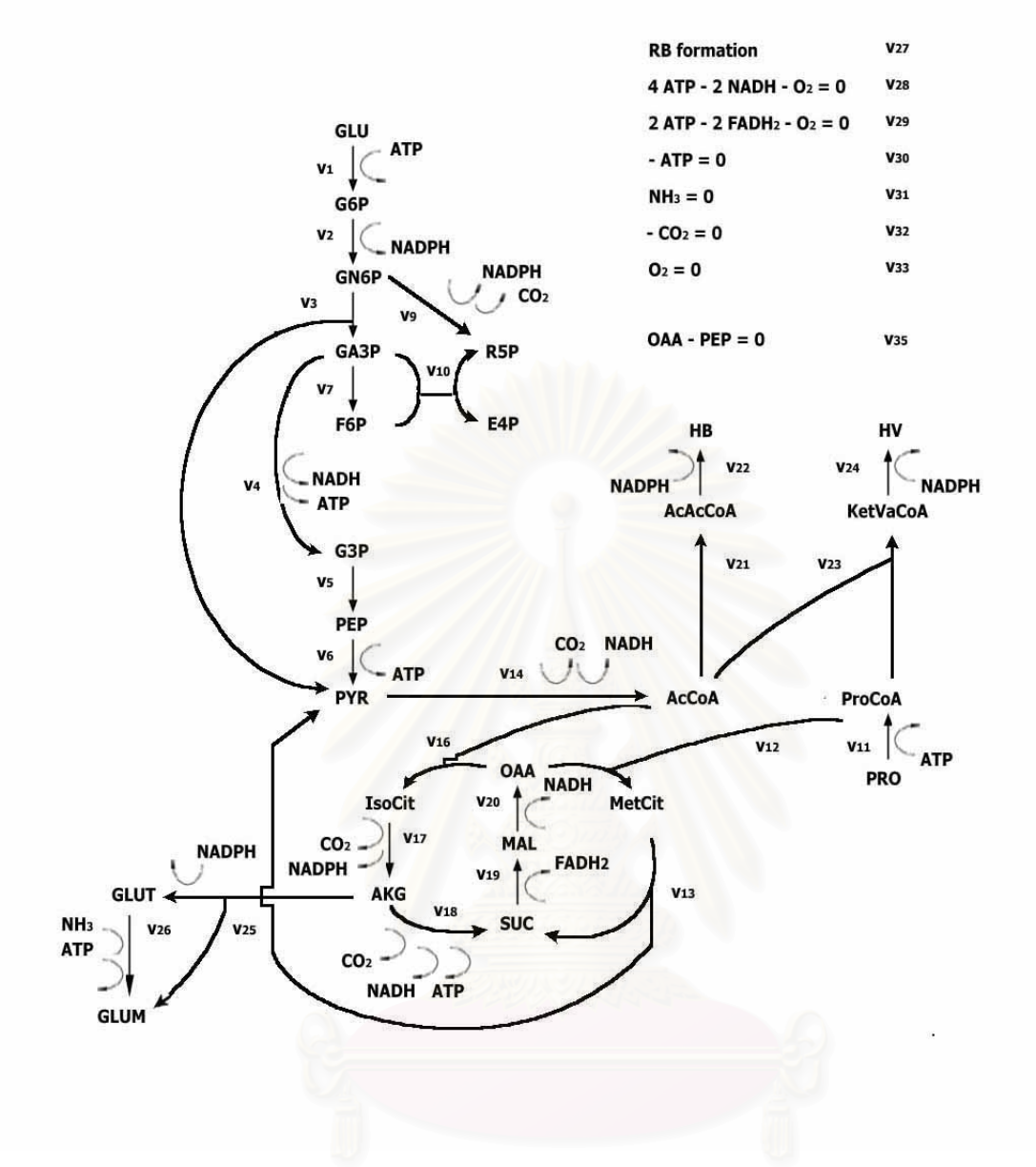


Figure 5.2-4: Proposed thermodynamically-feasible metabolic network when *Ralstonia eutropha* NCIMB 11599 is growing on a mixture of glucose and propionic acid where NADPH-linked malic enzyme is absent.

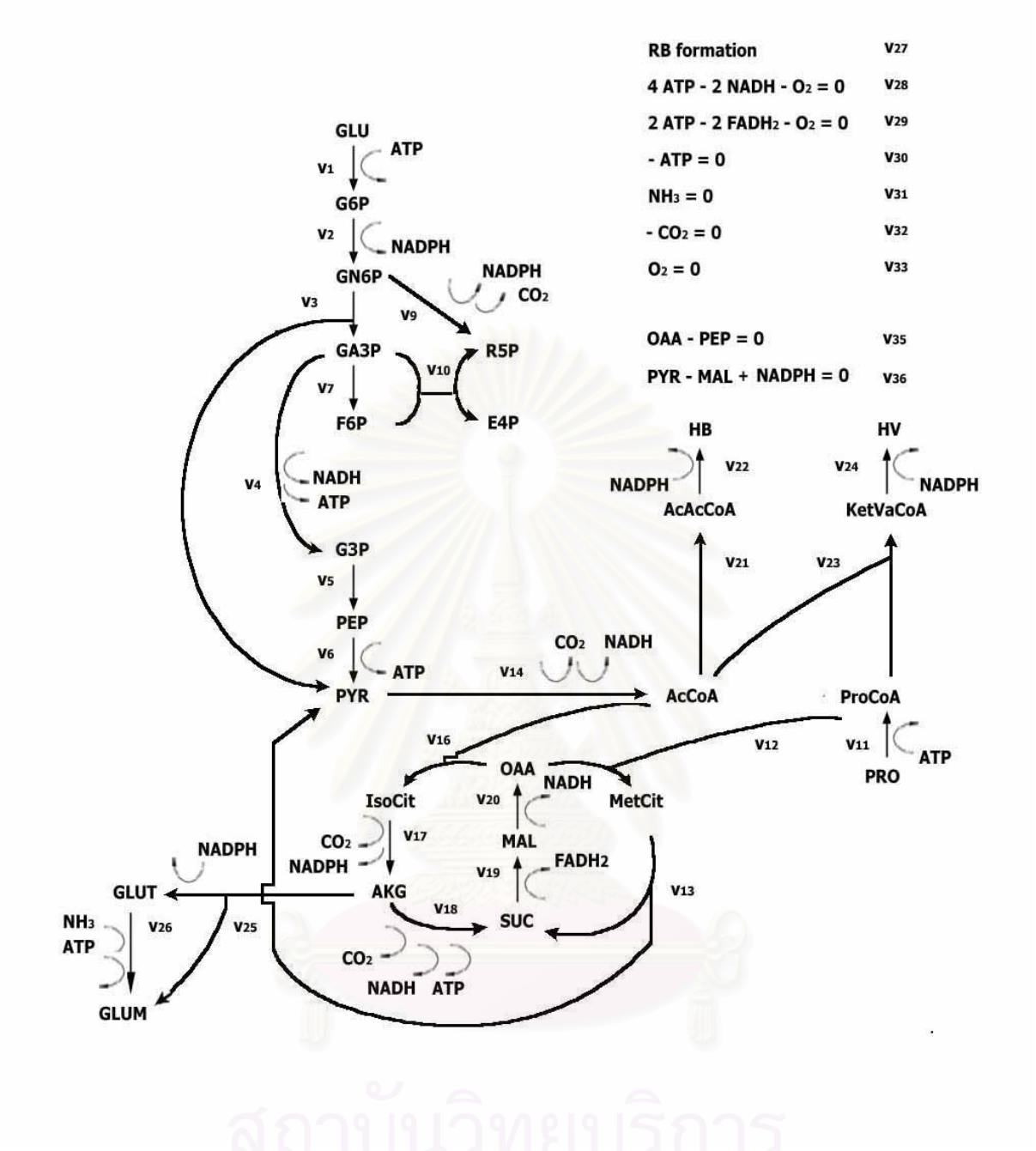


Figure 5.2-5: Proposed thermodynamically-feasible metabolic network when *Ralstonia eutropha* NCIMB 11599 is growing on a mixture of glucose and propionic acid where NADPH-linked malic enzyme is present.

5.2.6 Summary

From the application of metabolic flux analysis to identify metabolic pathways in proposed metabolic networks, the following aspects can be summarized.

- The operation of cyclic mode Entner-Doudoroff (ED) pathway is not affected by variation of molar carbon-to-nitrogen ratio. But it is markedly affected by variation of molar fraction of propionic acid in the reaction medium since it was found to operate only when glucose is available. This is crucially concluded based on the knowledge of biochemistry.
- Cyclic mode Entner-Doudoroff (ED) pathway fully operates only when *Ralstonia eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source (Molar fraction of propionic acid in the reaction medium is equal to 100%). But its function is to serve as a gluconeogenesis. Moreover, 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (represents as v_3) should be omitted in the metabolic network since its function is to serve for glycolysis.
- But when *R. eutropha* NCIMB 11599 is growing on either glucose or a mixture of glucose and propionic (Molar fraction of propionic acid in the reaction medium is not equal to 100%), cyclic mode of ED pathway is not fully active. Phosphoglucoisomerase (represents as v_8) could be considered inactive in comparison to all other enzymes in the ED pathway.
- Transhydrogenation (represents as v_{34}) is seemingly not active in *R. eutropha* NCIMB 11599 growing under all conditions within the range of study.
- Phosphoenolpyruvate carboxykinase (represents as v_{35}) is apparently the only route of anaplerotic pathway in *R. eutropha* NCIMB 11599 growing under all conditions within the range of study. Its activity is totally affected by variations of molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium.
- Activity of NADPH-linked malic enzyme (represents as v_{36}) is affected by variations of molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium. Accordingly, it is active in *R. eutropha* NCIMB 11599 growing on propionic acid as a sole carbon and energy source (Molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium are equal to 102 and 100%). In addition, it is also active if *R. eutropha* NCIMB 11599 is growing on an equimolar mixture of glucose and propionic acid under the utmost condition where severe nitrogen limitation is achieved (Molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium are equal to 200 and 50%). Seemingly, it is inactive at all other conditions of the remaining fermentation protocols.

Table 5.2-2: Summary of flux distributions for all fermentation protocols.

Flux	C30 1	C30 2	C30 3	C30 4	C30 5	C29 6	A14 7	B13 8	C30 9	C30 10	C30 11	C30 12	C30 13
v1	87.27	89.46	62.19	62.75	86.24	78.28	100.00	0.00	74.36	75.23	68.12	75.90	57.10
v11	12.73	10.54	37.81	37.25	13.76	21.72	0.00	100.00	25.64	24.77	31.88	24.10	42.90
v22	18.45	35.90	9.63	10.62	10.49	45.99	68.88	22.16	18.55	32.88	19.16	12.85	11.26
v24	6.96	9.83	5.65	12.08	4.74	11.74	0.00	11.77	12.08	26.68	10.63	12.35	4.98
v27	62.10	30.64	64.18	42.56	40.49	58.06	42.75	23.20	34.36	12.42	61.71	41.14	72.60
v31	32.59	17.40	22.95	14.47	130.93	24.17	18.09	14.94	15.84	19.59	22.18	11.72	33.09
v2	81.07	83.19	56.96	56.29	83.11	77.06	98.87	1.25	67.21	72.55	64.98	67.67	54.48
v3	76.09	79.66	52.20	52.13	80.16	73.93	96.48		63.21	71.08	61.03	62.99	50.19
v4	62.37	66.48	40.39	38.31	73.07	70.28	93.32	-4.94	48.20	65.47	53.44	45.67	43.41
v5	51.42	60.02	29.47	30.07	66.23	61.56	86.83	-8.42	40.91	62.80	43.56	37.04	32.16
v6	25.26	35.75	11.73	8.70	-2.61	20.52	81.62	-66.13	15.15	46.25	32.71	10.90	19.48
v7	3.71	3.97	3.04	4.01	1.80	0.41	0.45	1.90	4.52	1.70	1.67	5.20	1.25
v8								1.74					
v9	1.72	-0.22	2.17	0.44	1.43	3.13	2.24	1.25	-0.28	-0.13	2.72	-0.23	3.55
v10	3.87	2.98	3.61	3.39	2.21	2.09	1.61	0.84	3.38	1.25	2.84	3.94	2.98
v12	14.08	4.16	40.99	30.42	14.63	18.66	0.00	91.70	17.41	-0.54	30.11	16.44	48.55
v13	10.81	0.42	38.40	26.71	13.12	18.66	0.00	91.70	13.13	-2.14	28.87	11.52	47.82
v14	92.95	105.29	82.87	73.63	78.45	131.67	165.92	71.65	79.62	110.87	104.53	71.31	96.58
v16	40.85	17.23	50.33	33.05	48.04	23.60	28.00	13.82	23.15	15.72	49.60	24.78	62.84
v17	37.59	13.48	47.74	29.33	46.53	23.60	27.84	13.82	18.87	14.11	48.36	19.87	62.10
v18	38.82	11.30	55.18	32.64	-6.89	30.96	32.97	14.12	18.18	6.86	56.71	22.88	69.15
v19	48.00	9.84	92.28	57.49	5.47	49.62	32.90	105.82	29.16	3.91	84.97	31.95	116.60
v20	46.37	7.96	90.99	55.63	4.71	14.63	32.82	53.17	27.02	3.11	84.35	29.50	116.23

Table 5.2-2 (cont.): Summary of flux distributions for all fermentation protocols.

Flux	C30 1	C30 2	C30 3	C30 4	C30 5*	C29 6*	A14 7*	B13 8	C30 9	C30 10	C30 11	C30 12	C30 13
v21	15.12	35.11	5.86	8.92	8.06	41.65	63.63	20.43	17.69	32.60	15.04	11.74	6.12
v23	3.61	9.04	1.88	10.38	2.31	7.40	0.00	10.04	11.23	26.39	6.51	11.23	-0.15
v25	43.76	23.32	39.46	26.80	83.07	36.24	26.90	17.12	24.36	15.76	37.38	25.43	47.12
v26	37.93	20.88	29.85	20.17	115.83	28.68	21.44	15.86	20.39	18.95	28.18	18.27	38.61
v28	129.95	100.29	144.72	106.74	80.98	132.82	169.18	70.62	91.87	95.09	159.14	91.10	174.01
v29	24.00	4.92	46.14	28.74	2.73	24.81	16.45	52.91	14.58	1.96	42.48	15.98	58.30
v30	301.11	277.75	384.88	269.04	10.70	335.39	620.40	120.14	216.58	332.86	482.55	188.43	507.66
v32	165.98	117.05	191.60	129.85	64.11	201.50	237.21	103.06	103.51	119.84	221.41	103.03	241.80
v33	153.95	105.21	190.87	135.49	83.71	157.64	185.63	123.53	106.45	97.05	201.62	107.08	232.31
v34													
v35	21.31	20.79	13.11	17.31	65.98	38.02	2.92	56.50	21.84	15.10	7.03	21.54	8.53
v36						34.99		52.65					

5.3 Estimation of non-measured rates and reconciliation of measured rates

As a bonus of application of metabolic flux analysis, non-measured and measured rates are successfully estimated. This can be easily obtained after performing flux calculation. All estimated non-measured and measured rates of all fermentation protocols are collectively summarized in Appendix D.

Accordingly, there are two non-measured rates that can be obtained directly from flux estimates. The first one – carbondioxide evolution rate – is obtained as the estimated v_{32}. While the latter – oxygen uptake rate – is taken from v_{33}. Estimated values of carbondioxide evolution and oxygen uptake rates are ranged between 0.18-0.58 and 0.19-0.56 m mol / g RB / h, respectively. Additionally, values of respiratory quotient (RQ), defined as the ratio of carbondioxide evolution rate to oxygen uptake rate, are also estimated for all fermentation protocols. Estimated values of RQ are ranged between 0.8-1.3. (See *Table D14* for all estimated values of carbondioxide evolution rate, oxygen uptake rate, and respiratory quotient)

Unfortunately, values of carbondioxide evolution rate, oxygen uptake rate, and RQ are rarely stated in the literature. Thus, it is difficult to discuss whether the estimated values are well accepted. However, as far as we know, our range of RQ is satisfactorily in the range reported by Joo (1998), which is about 0.5-1.2. (As we approximately speculated from his report)

As for the measured rates, metabolic flux analysis could provide the set of reconciled values. These sets of measured rate values are usually more reliable than the experimentally-measured ones. Differences between the estimated and experimentally-measured values could spot another crucial information. Interestingly, it could suggest the source of errors resulted from model and measurement inconsistencies. Correspondingly, it is found that most of the uptake rates are underestimated, while most of the formation rates are overestimated. (See *Table 5.3-1* for all estimated values of measured rates)

Underestimation of the uptake rates might be resulted from either incomplete metabolic network models or measurement errors. As the first category, some pathways might be overlooked. Speaking of which, propionic acid might be degraded by an additional pathway, for instance, methylmalonyl coenzyme A pathway. But this might not be the case since the differences between the estimated and measured rates are quite small and, seemingly, within the range of measurement errors. Still, the proposed metabolic networks can describe the metabolic behavior quite well.

On the other hand, the overestimation of formation rates could reflect reliability of measurement methods, especially 3HB and 3HV formation rates. Inconsistency in metabolic network model might not contribute to that great extent since the proposed metabolic pathway for 3HB and 3HV formations is well established. Furthermore, the model has been widely used in metabolic flux analysis and yielded satisfactory results. [Shi *et al.*, 1997; Shi *et al.*, 1999; Wong *et al.*, 1999; Yu and Wong, 2001 for 3HB formation route and Chanprateep *et al.*, 2001 for 3HB and 3HV formation routes] Clearly, 3HB and 3HV formation rates should be revisited as the experimentally-measured values are quite far different from the estimated values. Specifically, the extraction process (to extract the polymer which is stored inside the cell out of the cell) needs to be carefully looked around as it is likely the major cause of measurement errors.

As well, the overestimation of formation rates might be resulted from the incompleteness of the proposed metabolic network models. Considered as the most probable

candidates, other by-product formations should be included in the model as soon as they are experimentally detected in a significant level.

Another interesting figure that can be computed from estimated non-measured and reconciled measured rates are carbon recovery. All estimated carbon recovery values are quite acceptable as they are approaching to 100% recovery. This implies that the proposed metabolic network of each fermentation protocol can describe the metabolic behavior of interest well and no artifact is included in the network.

However, for FB05 the carbon recovery (52.21%) is somewhat low. This might be resulted from such inconsistencies in either measurement errors or model incompleteness. Specifically, it could be due to undetected secretion of certain by-products, such as pyruvate, acetate, lactate, or citrate. Measurement of other secreted by-products should be carried out to check whether this is the case.

As already mentioned in the literature review, secretions of such intracellular metabolites can be observed when *R. eutropha* is growing in the oxygen-limited conditions. Dissolved oxygen tension in all Kittipar's fermentation protocols were kept at such high values to ensure the oxygen limitation could not be achieved. Thus, this could not be used for explaining the secretion behavior.

However, the above foreseeing might be supported by Joo's idea. [Joo, 1998] He suggested that the secretion might be explained by the bottlenecking of the respiratory capability. Supposing that the carbon influx is higher than the cell's respiratory capability, secretion of by-products is likely to occur. The larger the carbon influx, the more secretion of by-products is expected. In our results, such high value of carbon influx is observed for FB05 with intriguingly low RQ value. Consequently, Joo's idea might be suitably exploited to explain why the carbon recovery of FB05 is that low.

While for FB03, FB06, FB07, FB08, FB11, and FB13, the carbon recovery values are tolerably exceeding the limit of 100%. This might be caused by overestimation of certain formation rates, especially 3HB and 3HV formation rates. As already mentioned, 3HB and 3HV formation rates are overestimated comparing to those that are experimentally measured.

In conclusion, metabolic flux analysis could provide another interesting insight, that is, it yields all estimated non-measured rates and also reconciled measured-rates. These values, technically, could be inspected to check the degree of inconsistencies resulting from either model or measurement errors.

In this thesis, the estimated respiratory quotient (RQ) is used for comparing with other work. It appears that the estimated RQ ranges somewhat close to other work. This could imply that our proposed model is quite reliable.

As for the reconciliation of measured rates, it can be concluded that the differences between the reconciled and experimentally-measured rates are insignificantly small for all uptake and also residual biomass formation rates, but are somewhat noticeable for 3HB and 3HV formation rates. This suggests that these inconsistencies might be resulted from measurement errors. As such, it is recommended to look around the measurement methods of 3HB and 3HV. In addition, other by-product formations should be experimentally detected whether they are present in a significant level.

Table 5.3-1: Summary of estimated measured uptake rates of all fermentation protocols.

Run	Glucose uptake rate			Propionic acid uptake rate			Ammonium uptake rate		
	Measured	Estimated	% Different	Measured	Estimated	% Different	Measured	Estimated	% Different
FB01	48.4	45.8	-5.3	3.1	2.7	-11.4	10.5	12.6	19.6
FB02	44.7	42.0	-5.9	2.4	2.0	-15.2	4.8	6.0	24.9
FB03	33.7	31.7	-5.9	8.2	7.9	-3.3	6.0	8.6	43.0
FB04	17.0	15.6	-8.2	4.0	3.8	-4.7	1.6	2.6	65.0
FB05	51.9	50.6	-2.6	3.5	3.3	-5.3	62.8	56.3	-10.4
FB06	22.8	22.8	0.0	2.6	2.6	-0.1	4.2	5.2	22.9
FB07	54.7	54.6	-0.2				5.9	7.2	22.6
FB08				13.2	13.2	0.0	3.3	3.5	6.4
FB09	48.8	44.9	-8.0	6.9	6.4	-7.7	5.0	7.0	40.4
FB10	30.0	27.9	-7.0	3.9	3.8	-3.2	6.2	5.3	-14.0
FB11	33.0	32.1	-2.6	6.3	6.2	-1.9	5.6	7.7	37.0
FB12	57.2	52.1	-8.8	7.5	6.8	-9.2	2.6	5.9	126.9
FB13	21.6	21.2	-1.9	6.6	6.5	-0.9	7.5	9.0	20.0

Table 5.3-1 (cont.): Summary of estimated measured formation rates of all fermentation protocols.

Run	3HB formation rate			3HV formation rate			Residual biomass formation rate		
	Measured	Estimated	% Different	Measured	Estimated	% Different	Measured	Estimated	% Different
FB01	3.4	4.6	36.1	0.6	2.0	238.3	18.8	18.1	-3.6
FB02	6.8	8.1	18.6	1.1	2.6	133.6	7.8	8.0	2.6
FB03	1.4	2.3	67.7	0.5	1.6	220.0	19.5	18.2	-6.8
FB04	0.6	1.3	110.4	0.9	1.7	85.6	6.1	5.9	-3.6
FB05	2.3	2.9	27.9	0.8	1.5	92.5	8.6	13.2	53.4
FB06	6.4	6.4	0.0	1.9	1.9	0.0	10.3	9.4	-8.8
FB07	17.9	18.0	0.3				14.4	13.0	-10.0
FB08	3.4	3.4	-0.1	2.1	2.1	0.0	4.4	4.1	-5.9
FB09	3.5	5.4	53.1	1.9	4.1	113.7	11.4	11.5	1.1
FB10	4.0	5.8	45.8	2.6	5.5	111.5	14.7	2.6	-82.6
FB11	3.9	4.3	10.7	2.3	2.8	21.3	17.6	16.2	-8.1
FB12	1.8	4.2	134.1	1.9	4.7	147.9	16.0	15.7	-1.9
FB13	1.8	2.0	10.8	0.8	1.0	28.8	16.2	15.0	-7.6

5.4 Maximum theoretical molar fraction of 3HV monomeric unit in copolymer

5.4.1 Rationale

Another application of metabolic flux analysis is to determine maximum theoretical yield of product. As already cited, the ultimate aim of this research is to maximize molar fraction of 3HV monomeric unit in the copolymer. This section will cover how to obtain the maximum theoretical molar fraction of 3HV monomeric unit in the copolymer and its results.

The reason why we focus only on maximization of 3HV molar fraction in the copolymer is truly due to its commercial aspects. Practically, it is desirable that 3HV molar fraction be achieved in a high content to expand the applicability of the copolymer. The higher 3HV molar fraction, the broader applicability of the copolymer. Speaking of which, the higher 3HV fraction could render the copolymer less brittle and rigid. Also, it could reduce the melting point temperature of the copolymer so that the copolymer could be melt at a lower temperature. This lowering temperature is of significance, industrially, in polymer processing for product manufacturing process.

Actually, product productivity is the most vital parameter in a process improvement. However, in this research we do not stress on maximization of polymer productivity. The term 'productivity' is generally defined as the extent of product that could be obtained within the given time. Since the product of interest is the intracellular copolymer stored inside the residual cell, seemingly, the maximum productivity could be obtained by concurrently maximizing both the copolymer and residual cell concentrations. But this is not the case for our producing cell. In *Ralstonia eutropha*, synthesis of the copolymer could be enhanced by limitation of, for instance, nitrogen while keeping the carbon source in excess. This limitation directly reduces the synthesis of residual biomass. As a result, a dilemma inevitably occurs. As a matter of fact, we postpone the maximization of polymer productivity to be the next step of the improvement. Because it is such a state of art to optimally synthesize not only the copolymer but also residual biomass to maximize the copolymer productivity.

Practically, the procedure for determining a maximum yield value is conceptually based on ordinary optimization. This can be done by varying the set of solution that is still consistent with additional constraints until the optimum is obtained. For this manner, the objective function of our work is the maximum theoretical molar fraction of 3HV monomeric unit in the copolymer. While the set of solution is the set of estimated flux values. The equality constraints are the balances of intermediary metabolites according to the expression $\overline{\overline{T}}\,\overline{v} = \overline{0}$. (These constraints are governed by the fact that all intermediary metabolites are at their pseudo-steady state. See Chapter 3 for more details) In addition, the inequality constraints are the fact that all the estimated fluxes need to be non-negative according to the expression $\overline{v} \geq \overline{0}$. (These constraints are governed by their directionality of irreversibility and physiological sense. See section 5.2.2 for more details)

Another remark for the determination of maximum theoretical 3HV molar fraction is that only metabolic network case C30, where a mixture of glucose and propionic acid is used, will be persued. Since in metabolic network case A, where glucose is used as a sole carbon and energy source, no 3HV can be synthesized. As a result, it should be neglected. As for metabolic network case B, where propionic acid is used as a sole carbon and energy source, it is also not of interest. This is truly because of an industrial aspect. Efficient production process of the copolymer is suggested to employ two substrates. One is used as a precursor of

3HV monomeric unit and the other is for supporting cell growth and maintenance as well as NADPH production source. [Choi *et al*., 2003] If the 3HV-yielding substrate is used solely, such a portion might be lost through cell growth and maintenance as well as NADPH production. And finally, metabolic network case C29 is also not of interest. In fact, its maximum values are successfully computed but, as a matter of fact, they are somewhat close to the one obtained from metabolic network case C30. By this means, the values obtained from metabolic network case C30 are used further for the sake of comparison with the experimental values.

Maximum theoretical 3HV molar fraction can be considered as a benchmark where the product of interest can be obtained with the selected microbe. Interestingly, when comparing this value with the one computed from the experimental data, we can see how much environmental perturbation could affect the yield. If the experimental value is approaching the theoretical one, this implies that only environmental perturbation could be efficient enough and the improvement might not be subsequently needed to perform. But, in reality, the experimental value is much lower than the theoretical one. Hence, only environmental perturbation might not be sufficient to improve the process. Inevitably, metabolic engineering is needed.

In the following context of this section, we will investigate whether metabolic engineering is to be asked for application in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis in *Ralstonia eutropha* NCIMB 11599

5.4.2 Determination of maximum theoretical 3HV molar fraction

In this research, the method of how to determine maximum theoretical 3HV molar fraction is exactly the same as the method suggested by Stephanopoulos *et al*. (1998). As a brief description, there are eight consecutive steps in determining the maximum theoretical 3HV molar fraction as shown in *Figure 5.4-1*.

First, declaration of substrates, products, and by-products is to be made. As already mentioned, the substrate of interest is a mixture of glucose and propionic acid. The desired product is 3HV monomeric unit, while the by-product of interest is solely 3HB monomeric unit. It is noted that residual biomass and ammonium are not taken into account since the maximal value is obtained when carbon flux is not lost through biomass formation. [Stephanopoulos *et al*., 1998]

Then it is needed to set all formation rates except that of a desired product to be zero. As for substrate uptake rate, conventionally, it is set to 100. However, in this research there are two substrates of interest; glucose and propionic acid. We set overall uptake rate to 100 and let the uptake rate of glucose range from 10 to 90 and that of propionic acid range from 90 to 10 accordingly. Next, it is the time we perform flux calculation by running the programmed MATLAB m-file as we have previously done. [See section 5.1 for details on flux calculation]

Next, technically, maximum theoretical yield can be obtained by continuing increasing the yield value as soon as infeasible flux distribution results do not appear. Infeasible flux distribution results are recognized as some flux values violate either their directionality of irreversible or physiological function.

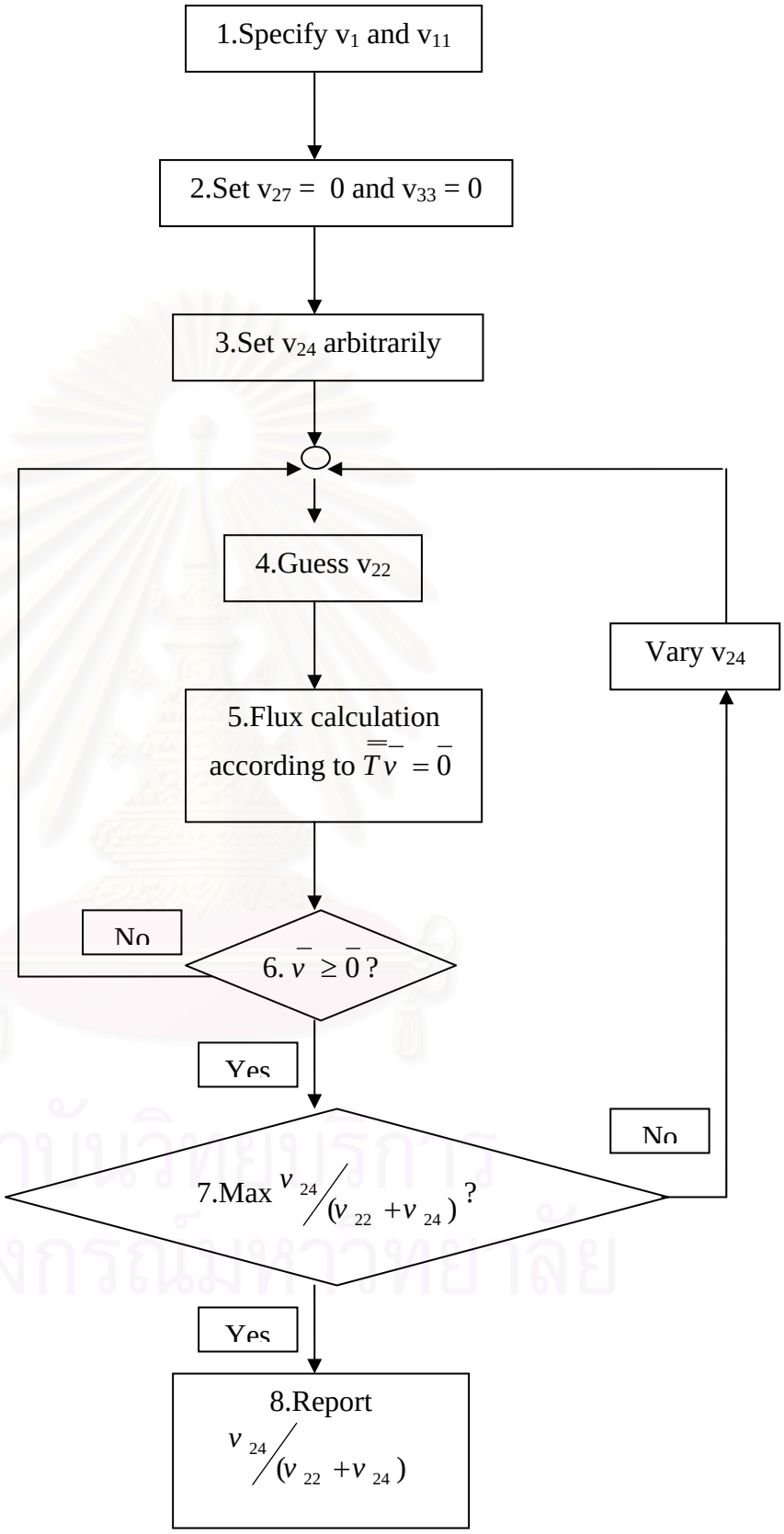


Figure 5.4-1: Procedure for the determination of maximal theoretical 3HV molar fraction in the copolymer.

Summarized in *Table 5.4-1* are the estimated values of maximum molar fraction of 3HV monomeric unit in the copolymer under various molar ratios of glucose uptake rate to propionic acid uptake rate. (Details on the corresponding flux distributions can be consulted in Appendix D) As molar ratio of glucose uptake rate to propionic acid uptake rate decreases, molar fraction of 3HV monomeric unit in the copolymer increases dramatically. Thus, if we desire to get higher molar fraction of 3HV monomeric unit in the copolymer experimentally, it is recommended to maintain uptake rate of propionic acid to be higher than that of glucose.

Unfortunately, this cannot be realized in reality since propionic acid is much more toxic than glucose and the microbe is apt enough to control the ratio of uptake rates. This is clearly noticed in *Table A2* where specific glucose uptake rates were found to be in the range of 1.35 to 7.66 times higher than specific propionic acid uptake rates on the molar basis. According to Kasipar's experimental data, molar fraction of 3HV monomeric unit in the copolymer has not exceeded the value of 53%. (See *Table D15*) But as it can be clearly seen in *Table 5.4-1*, the maximum theoretical value is 60.00% if the molar ratio of glucose uptake rate to propionic acid uptake rate is set at 5.74:4.26. (The ratio of 5.74:4.26 is selected for comparison since it is equal to 1.35, the experimental data) Hence, there is more room for improvement of the microbe to incorporate more 3HV fraction into the copolymer.

Table 5.4-1: Maximum theoretical molar fraction of 3HV monomeric unit in the copolymer at various molar ratios of glucose uptake rate to propionic acid uptake rate.

Characteristic	Molar ratio of glucose uptake rate to propionic acid uptake rate			
	9:1	7:3	5.74:4.26	5:5
3HV fraction	10.71	37.50	60.00	75.00

5.5 Identification of principal nodes and possible bottlenecks in proposed metabolic networks

5.5.1 Rationale

Before commencing this section, the term ‘flux split ratio’ is to be recognized. According to Vallino and Stephanopoulos (1993), flux split ratio is defined as the carbon flux channeled through a branch normalized by the total flux into the node.

Although metabolic networks contain a large number of nodes, it is usually the case that flux split ratios at only a few nodes actually change as the yield of the products changes. These nodes are referred to as principal nodes as they directly affect product yield. The split ratios of the remaining nodes are relatively unaffected and do not merit further investigation. [Stephanopoulos *et al*., 1998]

To locate the principal nodes in a network, the products, by-products, and substrates are first identified. For this research, we aim at enhancement of molar fraction of 3HV monomeric unit in copolymer. Thus, desired product that needs to be maximized is, inevitably, 3HV monomeric unit leaving 3HB monomeric unit and residual biomass as by-products. While the substrate is a mixture of glucose and propionic acid. (See topic 5.4 why the mixture case is merely focused)

Then principal nodes are identified by systematic variation of the product yield and observation of the flux split ratios of various nodes. This can be done by two approaches. The first one is indeed accommodated through theoretical analysis, whereas the second is perceived as experimental analysis.

Various studies have exploited the latter approach to identify the principal nodes since the identified nodes are correspondingly supported by experimental results. As a matter of fact, frequently, they can do so without any complaints as they only make a variation in one variable. Unfortunately, in this research there are two variables to be varied so as to identify the principal nodes. Analysis of experimental flux split ratios is difficult as there might be an interaction effect between the two variables. However, this might give another intriguing aspects involving with the identified principal nodes as will be covered in the succeeding topic.

As well, we apply the theoretical analysis for the identification of principal nodes as achieved by Vallino (1991). [Stated in Stephonopoulos *et al*., 1998] Interestingly, all principal nodes obtained by both approaches are compared and discussed.

Once, the principal nodes can be identified, possible bottlenecks can be easily pointed out. These bottlenecks then are the focus of the following section, suggestion of rational modifications for metabolic engineering.

5.5.2 Theoretical approach

This section will cover the procedure how we could identify the principal nodes in our metabolic network by the theoretical approach as well as the obtained results. The procedure is somewhat similar to the procedure for determination of maximal 3HV fraction as shown in *Figure 5.4-1* except for a few points.

Like the procedure for the determination of maximal 3HV fraction, the process starts with the specification of the uptaking ratio of glucose to propionic acid (Step 1 in *Figure 5.4-1*). For example, the ratio is set at 7:3.

Then we set the biomass formation and ammonium uptake rate to zero (Step 2 in *Figure 5.4-1*).

After that, we vary 3HB and 3HV formation rates so as to vary the 3HV molar fraction in the copolymer (Step 3-6 in *Figure 5.4-1*). To achieve so, the following variations of 3HB and 3HV formation rates are made as 70:10, 60:20, and 50:30, respectively. As a result, the 3HV fraction of each variation is as 12.50%, 25.00%, and 37.50%, respectively.

Next, unlike the procedure in *Figure 5.4-1*, we inspect which flux split ratio is changed according to the change in 3HV molar fraction. (See Appendix D for more details on the corresponding flux split ratios at all branch-points for various scenario cases)

Apparently, at the acetyl coenzyme A (AcCoA) node, there are changes in flux split ratios of v_{21}/v_{14}, v_{23}/v_{14}, and v_{16}/v_{14}. Likewise, at the propionyl coenzyme A (ProCoA) node, there are changes in flux split ratios of v_{12}/v_{11} and v_{23}/v_{11}. In contrast, at all other nodes, there is no change in any flux splits. (The flux split ratios are either 100 or 0 at all conditions) Consequently, it can be concluded that there are two principal nodes in the proposed network; namely, AcCoA and ProCoA.

To further investigate possible bottlenecks, only the values at the molar ratio of glucose uptake rate to propionic acid uptake rate of 7:3 are selected on purpose as depicted in *Figure 5.5-1* and *Figure 5.5-2*. It is noteworthy to state that, technically, the values at all other conditions can also be used for the analysis and certainly yield the same conclusion.

According to *Figure 5.5-1*, at AcCoA node, molar fraction of 3HV monomeric unit can be increased dramatically by increasing v_{16} and v_{23} while decreasing v_{21}. Apparently, increasing of v_{23} assuredly enhances molar fraction of 3HV monomeric unit since this flux represents the condensation step of AcCoA and ProCoA to yield 3-ketovaleryl coenzyme A (KetVaCoA), which is finally further polymerized to 3HV monomeric unit. Concerning v_{21}, it can be realized as the competing route to formation of 3HB monomeric unit. Thus, it is recommended to decrease v_{21} while at the same time increase v_{23}. However, this cannot be done easily since both are catalyzed by the same set of enzymes – 3-ketothiolase, acetoacetyl coenzyme A reductase, and polymerase. Another striking feature of increasing molar fraction of 3HV monomeric unit is at the increasing of v_{16}. At the first glance, this might be against our intuitive. But this might be the case because the incremental amount of v_{16} could provide more NADPH as v_{16} directly relates to NADPH-linked isocitrate dehydrogenase, represented as v_{17}. Apparently, as the molar ratio of glucose uptake rate to propionic acid uptake rate is increasing, the flux split ratio of v_{16}/v_{14} needs to be increasing as well. This indicates that NADPH production from v_2 is insufficient. Thus, more NADPH production is demanded to meet the requirement of NADPH in copolymer synthesis. As such, it asks for the operation of isocitrate dehydrogenase as an additional NADPH production site.

Regarding ProCoA node, molar fraction of 3HV monomeric unit can be increased markedly by decreasing v_{12} and increasing v_{23}. The reason why increasing v_{23} can enhance molar 3HV monomeric unit is exactly the same as above. While decreasing v_{12} is a result of increasing v_{23} as their summation should be equal to the incoming flux of v_{11}.

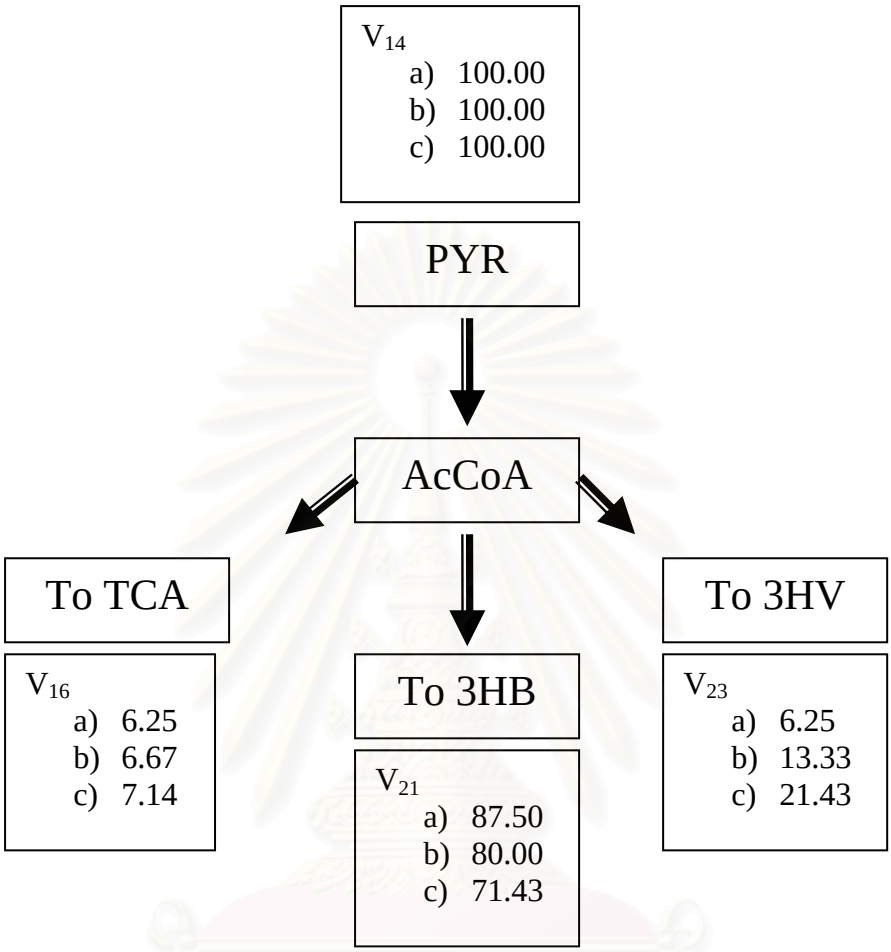


Figure 5.5-1: Theoretical flux split ratio at acetyl coenzyme A (AcCoA) node. The values are obtained at the molar ratio of glucose uptake rate to propionic acid uptake rate of 7:3. The values in each case are the corresponding flux split ratio when the theoretical molar fractions of 3-hydroxyvalerate monomeric unit are as follows; a) 12.50% b) 25.00% and c) 37.50%.

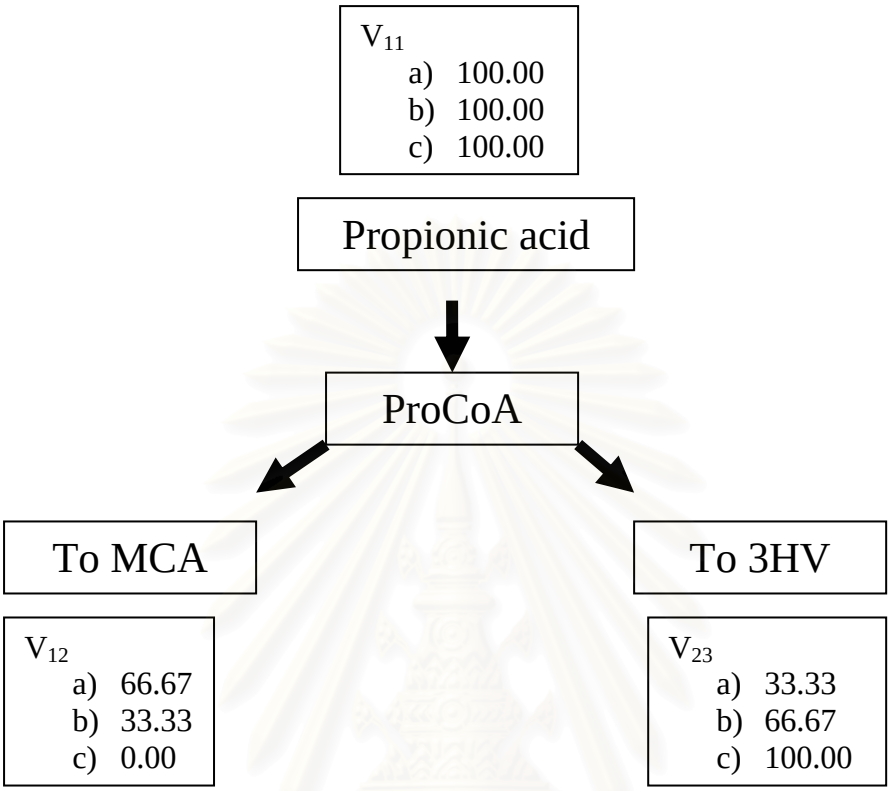


Figure 5.5-2: Theoretical flux split ratio at propionyl coenzyme A (ProCoA) node. The values are obtained at the molar ratio of glucose uptake rate to propionic acid uptake rate of 7:3. The values in each case are the corresponding flux split ratio when the theoretical molar fractions of 3-hydroxyvalerate monomeric unit are as follows; a) 12.50% b) 25.00% and c) 37.50%.

5.5.3 Experimental approach

As already mentioned, two key parameters are varied experimentally; molar carbon-to-nitrogen ratio (C/N) and molar fraction of propionic acid (%P) in the reaction medium. So it is wise to investigate the effect of each parameter on the principal nodes one at a time. And then, hopefully, we might see the interaction effects, if there might be, from both parameters.

To examine the effect of molar carbon-to-nitrogen ratio, we select two pairs of the experimental data on purpose. The first pair is that of the fermentation protocol FB01 and FB02, while the second pair is that of FB03 and FB04. Details on flux split ratios of both pairs are present in *Table 5.5-1*.

On the contrary, we cross the stated pairs to figure out the effect of molar fraction of propionic acid in the reaction medium. Specifically, the data of FB01 are compared to that of FB03 and the data of FB02 are compared to that of FB04. Details on flux split ratios of both pairs are present in *Table 5.5-2*.

According to *Table 5.5-1*, at molar %P of 14.7, the following flux split ratios are markedly changed in response to the variation of molar C/N from 32.7 to 171.3; namely, v_{27}/v_1, v_9/v_2, v_{27}/v_3, v_{27}/v_7, v_{27}/v_4, v_{27}/v_5, $v_{27}/(v_3+v_6+v_{13})$, v_{16}/v_{14}, v_{21}/v_{14}, v_{23}/v_{14}, v_{27}/v_{14}, v_{23}/v_{11}, and v_{12}/v_{11}. These flux split ratios are located at the following principal nodes; glucose-6-phosphate (G6P), 6-phosphogluconate (GN6P), glyceraldehyde-3-phosphate (GA3P), fructose-6-phosphate (F6P), 3-phosphoglycerate (G3P), phosphoenolpyruvate (PEP), pyruvate (PYR), acetyl coenzyme A (AcCoA), and propionyl coenzyme A (ProCoA).

But when we focus on the effect of molar C/N at molar %P of 85.4, there are only a few changing flux split ratios left; namely, v_9/v_2, v_{23}/v_{14}, and v_{23}/v_{11}. As a result, there are only three principal nodes; namely, GN6P, AcCoA, and ProCoA.

The above findings suggest that only GN6P, AcCoA, and ProCoA might be the only principal nodes that are directly influenced by molar C/N. Whereas all the other nodes might appear because of the interaction effect resulting from both molar C/N and %P.

According to *Table 5.5-2*, at molar C/N of 171.3, the following flux split ratios are markedly changed in response to the variation of molar %P from 14.7 to 85.4; namely, v_9/v_2, v_{27}/v_3, v_{27}/v_4, v_{27}/v_5, v_{16}/v_{14}, v_{21}/v_{14}, v_{23}/v_{11}, v_{12}/v_{11}, and $v_{12}/(v_{20}+v_{35})$. These flux split ratios are located at the following principal nodes; GN6P, GA3P, G3P, PEP, AcCoA, ProCoA, and oxaloacetate (OAA).

But when we focus on the effect of molar %P at molar C/N of 32.7, there are only a few changing flux split ratios left; namely, v_{23}/v_{14} and v_{23}/v_{11}. As a result, there are only three principal nodes; namely, AcCoA and ProCoA.

The above findings suggest that only AcCoA and ProCoA might be the only principal nodes that are directly influenced by molar %P. Whereas all the other nodes might appear because of the interaction effect resulting from both molar C/N and %P.

Table 5.5-1: Effects of molar carbon-to-nitrogen ratio on flux split ratios.

Description	Protocol	FB01	FB02	%Diff	FB03	FB04	%Diff
	C/N	32.7	171.3		32.7	171.3	
	%P	14.7	14.7		85.4	85.4	
	HV fraction	27.40	21.51	21.50	36.95	53.22	30.57
Node	Flux split ratio	FB01	FB02	%Diff	FB03	FB04	%Diff
G6P	V2/v1	92.89	93.00	0.11	91.60	89.70	2.07
	v27/v1	1.49	0.72	51.87	2.17	1.42	34.28
GN6P	v3/v2	93.86	95.76	1.98	91.64	92.61	1.05
	v9/v2	2.13	-0.26	112.36	3.82	0.79	79.33
GA3P	v4/v3	81.97	83.46	1.78	77.38	73.49	5.03
	v7/v3	9.74	9.95	2.16	11.65	15.39	24.31
	v10/v3	5.09	3.74	26.57	6.91	6.50	5.94
	v27/v3	1.06	0.50	52.88	1.60	1.06	33.60
F6P	v10/v7	104.48	75.06	28.16	118.70	84.50	28.81
	v27/v7	11.73	5.41	53.89	14.78	7.43	49.74
G3P	v5/v4	82.44	90.27	8.67	72.96	78.49	7.05
	v27/v4	14.93	6.91	53.71	23.83	16.66	30.08
PEP	v6/v5	49.11	59.57	17.55	39.80	28.93	27.32
	v27/v5	6.28	2.65	57.73	11.32	7.36	35.01
	v35/v5	41.44	34.65	16.39	44.48	57.56	22.71
PYR	v14/(v3+v6+v13)	82.87	90.90	8.83	80.99	84.12	3.72
	v27/(v3+v6+v13)	15.67	7.49	52.23	17.75	13.76	22.48
AcCoA	v16/v14	43.95	16.36	62.77	60.73	44.88	26.09
	v21/v14	32.53	66.68	51.22	14.14	24.22	41.60
	v23/v14	3.89	8.59	54.74	2.27	14.10	83.89
	v27/v14	24.99	10.88	56.45	28.96	21.62	25.36
ProCoA	v23/v11	28.39	85.75	66.90	4.98	27.88	82.14
	v12/v11	110.64	39.50	64.30	108.40	81.66	24.67
AKG	v18/(v17+v27)	46.10	30.96	32.84	57.52	53.26	7.40
	v25/(v17+v27)	51.96	63.92	18.71	41.14	43.72	5.90
OAA	v12/(v20+v35)	20.81	14.48	30.39	39.38	41.70	5.57
	v16/(v20+v35)	60.36	59.92	0.72	48.35	45.31	6.29
	v27/(v20+v35)	16.42	19.07	13.89	11.04	10.44	5.36
GLUT	v26/v25	43.33	44.76	3.19	37.82	37.63	0.49
	v27/v25	59.03	54.64	7.43	67.65	66.07	2.33

Note: - %Diff is defined as (larger value – smaller value) / larger value * 100.
- Flux split ratio present in the table is written in short. It does not include the stoichiometry. For instance, v_{27}/v_1 is the short form of 0.021 * v_{27} / v_1.
- Highlighted are the values larger the criterion value of 50%.

Table 5.5-2: Effects of molar fraction of propionic acid on flux split ratios.

Description	Protocol	FB01	FB02	%Diff	FB03	FB04	%Diff
	C/N	32.7	32.7		171.3	171.3	
	%P	14.7	85.4		14.7	85.4	
	HV fraction	27.40	21.51	21.50	36.95	53.22	30.57
Node	Flux split ratio	FB01	FB03	%Diff	FB02	FB04	%Diff
G6P	v2/v1	92.89	91.60	1.40	93.00	89.70	3.54
	v27/v1	1.49	2.17	31.05	0.72	1.42	49.50
GN6P	v3/v2	93.86	91.64	2.37	95.76	92.61	3.29
	v9/v2	2.13	3.82	44.29	-0.26	0.79	133.32
GA3P	v4/v3	81.97	77.38	5.61	83.46	73.49	11.95
	v7/v3	9.74	11.65	16.37	9.95	15.39	35.30
	v10/v3	5.09	6.91	26.38	3.74	6.50	42.53
	v27/v3	1.06	1.60	33.62	0.50	1.06	52.89
F6P	v10/v7	104.48	118.70	11.98	75.06	84.50	11.17
	v27/v7	11.73	14.78	20.63	5.41	7.43	27.19
G3P	v5/v4	82.44	72.96	11.50	90.27	78.49	13.05
	v27/v4	14.93	23.83	37.34	6.91	16.66	58.52
PEP	v6/v5	49.11	39.80	18.97	59.57	28.93	51.44
	v27/v5	6.28	11.32	44.55	2.65	7.36	63.93
	v35/v5	41.44	44.48	6.84	34.65	57.56	39.80
PYR	v14/(v3+v6+v13)	82.87	80.99	2.27	90.90	84.12	7.46
	v27/(v3+v6+v13)	15.67	17.75	11.72	7.49	13.76	45.60
AcCoA	v16/v14	43.95	60.73	27.63	16.36	44.88	63.54
	v21/v14	32.53	14.14	56.52	66.68	24.22	63.68
	v23/v14	3.89	2.27	41.55	8.59	14.10	39.11
	v27/v14	24.99	28.96	13.73	10.88	21.62	49.66
ProCoA	v23/v11	28.39	4.98	82.46	85.75	27.88	67.49
	v12/v11	110.64	108.40	2.02	39.50	81.66	51.63
AKG	v18/(v17+v27)	46.10	57.52	19.86	30.96	53.26	41.88
	v25/(v17+v27)	51.96	41.14	20.83	63.92	43.72	31.60
OAA	v12/(v20+v35)	20.81	39.38	47.16	14.48	41.70	65.27
	v16/(v20+v35)	60.36	48.35	19.90	59.92	45.31	24.39
	v27/(v20+v35)	16.42	11.04	32.80	19.07	10.44	45.24
GLUT	v26/v25	43.33	37.82	12.72	44.76	37.63	15.92
	v27/v25	59.03	67.65	12.74	54.64	66.07	17.30

Note: - %Diff is defined as (larger value – smaller value) / larger value * 100.
- Flux split ratio present in the table is written in short. It does not include the stoichiometry. For instance, v_{27}/v_1 is the short form of 0.021 * v_{27} / v_1.
- Highlighted are the values larger the criterion value of 50%.

When analyzing the experimental data to identify possible principal nodes, it is difficult to identify principal nodes that truly affect the 3HV molar fraction. This is because the two key parameters affect directly on the formation of 3HV and 3HB or even residual biomass, Speaking of which, certain nodes might have a direct effect only on 3HV formation. On the other hand, some might have a direct effect only on residual biomass. Or even some might directly influence on both 3HV and residual biomass formations.

In conclusion, principal nodes obtained by experimental analysis are depending upon the molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium. However, these nodes are under suspicion whether they have a direct effect on 3HV molar fraction. To elucidate this, we compare the experimental observations with that obtained by the theoretical analysis. As a result, acetyl coenzyme A (AcCoA) and propionyl coenzyme A (ProCoA) are believed to be the only possible principal nodes that directly influence 3HV molar fraction. The other nodes suggested by the experimental analysis might not only contribute to 3HV formation but also to residual biomass formation.

5.5.4 Summary

In summary, there are two obvious principal nodes in the proposed metabolic network where a mixture of glucose and propionic acid is used. These two nodes are at acetyl coenzyme A (AcCoA) and propionyl coenzyme A (ProCoA) nodes. Consequently, the fluxes around these two nodes are marked as possible bottlenecks for maximal synthesis of 3HV monomeric unit in the copolymer.

Theoretically, to achieve higher 3HV fraction, the following aspects are suggested to perform. However, it is remarked that the following regimes might not perceived when conducting an experiment. As such, experiments are needed to confirm the suggestions.

- The flux of AcCoA that condenses with ProCoA should be increased to directly enhance 3HV fraction in the copolymer.
- The flux of AcCoA that condenses with another AcCoA should be decreased to reduce 3HB formation.
- The flux of ProCoA that condenses with AcCoA should be increased by the same implication with the first suggestion.
- The flux of ProCoA that enters the methylcitric acid (MCA) cycle should be decreased.

5.6 Suggestion of rational modifications for metabolic engineering

As the ultimate aim of this research, after successfully performing metabolic flux analysis, such a suggestion should be drawn out as rational modifications for metabolic engineering. Specifically, these modifications can be achieved by either genetic engineering or enzyme manipulation.

5.6.1 Suggested strategies

Apparently, there are six strategies for metabolic engineering according to the possible bottlenecks suggested in the previous topic. The following are the summary of the strategies. As well, the targeted enzyme or gene corresponding to each strategy is illustrated in *Figure 5.6-1*.

- Engineering the active site of 3-ketothiolase for much more active with ProCoA comparing to AcCoA. This is supported by the study of Slater *et al*. (1998).
- Amplifying PHB synthase according to the claim of Jung *et al*. (2000).
- Fully or partially blocking isocitrate dehydrogenase enzyme as succeeded by Park *et al*. (1996).
- Fully or partially blocking methylcitrate lyase as suggested by Lee, I.Y. *et al*. (1996) and Bramer *et al*. (2002).
- Amplifying glucose-6-phosphate dehydrogenase enzyme to produce more NADPH. This is confirmed by Choi *et al*. (2003).
- Increasing intracellular ProCoA by feeding additional ProCoA-yielding substrate and/or introducing an additional ProCoA formation route according to the study of Aldor and Keasling (2001), Steinbuchel and Pieper (1992), and Gruys *et al*. (2003).

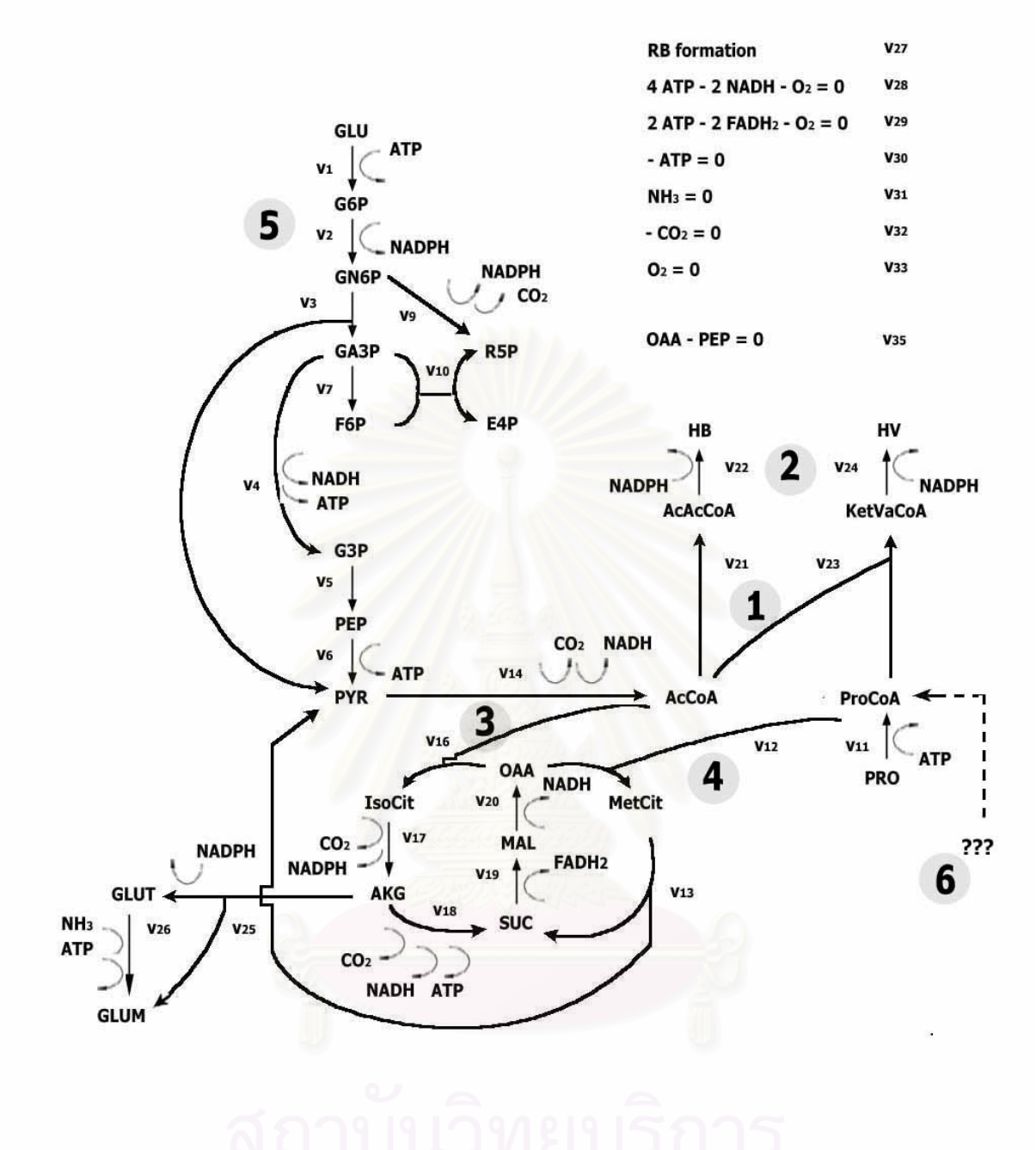


Figure 5.6-1: Targeted enzyme or gene for metabolic engineering.

5.6.2 Supporting works

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) biosynthesis proceeds through the action of only three enzymes; namely, 3-ketothiolase, acetoacetyl coenzyme A reductase, and PHB synthase. The genes encoding these enzymes are located in the same phbCAB operon. Apparently, these genes are essential in the copolymer biosynthesis. In addition, other factors could affect the biosynthesis. These factors include enzymes that are participated in the central metabolism, global metabolic regulation, as well as levels of precursors. This turns the modification by metabolic engineering not easy to be attained. To achieve the higher molar fraction of 3HV monomeric unit, the following publications are selected to be reviewed.

Basically, *R. eutropha* contains two 3-ketothiolases – enzyme A and enzyme B – that are able to act in the biosynthetic pathway to PHA synthesis. The major difference between these two enzymes is their substrate specificity. Enzyme A is a homotetramer of 44-kDa subunits and converts acetoacetyl coenzyme A (AcAcCoA) and 3-ketopentanoyl coenzyme A (but only at 3% relative activity in comparison to AcAcCoA). In contrast, enzyme B, a homotetramer of 46-kDa subunits, has a broader substrate specificity and cleaves AcAcCoA as well as 3-ketopentanoyl coenzyme A, 3-ketohexanoyl coenzyme A, 3-ketoheptanoyl coenzyme A, 3-ketooctanoyl coenzyme A, and 3-ketodecanoyl coenzyme A (30, 17, 19, 10, and 12% activity relative to AcAcCoA, respectively). Originally, it was thought that the major role of enzyme B is in fatty acid degradation while the primary role of enzyme A (PhbA) is in the biosynthesis of P(3HB). [Haywood *et al*., 1988] Recently, however, it has been shown that enzyme B is the primary source of 3HV monomeric unit for P(3HB-*co*-3HV) formation. [Slater *et al*., 1998] Hence, if we could engineer the active site of enzyme B to be much more than enzyme A, the higher molar fraction of 3HV monomeric unit might be achieved.

Doi *et al*. (1992) studied both the synthesis and degradation mechanisms of P(3HB-*co*-3HV) in *R. eutropha* by using ^{13}C-NMR. They proposed that acetoacetyl coenzyme A reductase is the key enzyme determining the rate of P(3HB-*co*-3HV) biosynthesis and the molar fraction of 3HV rather than PHB synthase. However, the above observations were accomplished in vitro by using purified individual enzyme. Hence, they cannot fully reflect the dynamic role of each enzyme related to P(3HB-*co*-3HV) biosynthesis in vivo. [Jung *et al*., 2000]

Instead, Jung *et al*. (2000) reported that the accumulation rate of P(3HB-*co*-3HV) in *R. eutropha* transformant was more closely connected with 3-ketothiolase or acetoacetyl coenzyme A reductase rather than PHB synthase. In contrast, the molar fraction of 3HV in the copolymer was more closely connected with PHB synthase rather than 3-ketothiolase. The fortification of 3-ketothiolase seems to be profitable in increasing P(3HB-*co*-3HV) accumulation rate. On the other hand, amplification of PHB synthase seems to be favorable for production of the copolymer containing high 3HV molar fraction.

Park *et al*. (1996) successfully isolated the isocitrate dehydrogenase leaky mutant *R. eutropha* RM-1 by mutagenesis. Compared to the parent strain, in the mutant the molar fraction of 3HV and its yield from propionic acid were increased due to the increased conversion of propionic acid to 3-hydroxyvaleryl coenzyme A rather than to AcCoA and carbondioxide.

Lee, I.Y. *et al*. (1996) developed a mutant strain of *R. eutropha* NCIMB 11599 which is unable to assimilate propionic acid for cell growth. Then the mutant and the parent strains were compared as to their ability to accumulate P(3HB-*co*-3HV) and their composition in two-staged flask and chemostat cultures. With the mutant, the fraction of 3HV and its yield from propionic acid were found to be significantly improved compared to those of the parent strain. As such, this research successfully diminished the flux of ProCoA to the methylcitric acid (MCA) cycle.

Bramer *et al*. (2002) reported that *Burkholderia sacchari* IPT101T mutant defective in prpC could synthesize P(3HB-*co*-3HV) containing high 3HV molar fraction. Apparently, prpC gene is believed to encode 2-methylcitrate synthase, the first enzyme in the methylcitric acid (MCA) cycle.

Choi *et al*. (2003) successfully engineered *R. eutropha* transformant co-amplifying phbC and NADPH generation-related zwf genes to synthesize P(3HB-*co*-3HV) from a mixture of fructose and valerate in a nitrogen-free minimal medium. The phbC gene cloned from *R. eutropha* H16 and/or zwf gene cloned from *E. coli* DH5α were introduced into the parent *R. eutropha* H16 to enhance the PHB synthase and/or glucose-6-phosphate dehydrogenase (G6PDH) activities, respectively. The amplified phbC gene alone influenced the intrinsic PHB synthase activity, which was 2.3 times higher compared to the parent strain. The amplified zwf gene alone had a direct influence on the G6PDH activity, which exhibited a significant 3.3-fold increase, while indirectly influencing the 3-ketothiolase and AcAcCoA reductase activities, which were 1.3 and 1.4 times higher, respectively. The enforcement of the zwf gene also made the NADPH level around 1.6 times higher. As a result, the biosynthesis of P(3HB-*co*-3HV) increased appreciably after the co-amplification of these two genes. As well, the 3HV molar fraction in the copolymer was effectively modulated. It was reported that the molar fraction of 3HV could be synthesized up to the level of 71.9%, which is remarkably high.

A recombinant strain of *Salmonella enterica serovar* Typhimurium was metabolically engineered by Aldor and Keasling (2001) to control the composition of P(3HB-*co*-3HV) with commercially desirable properties. A gene prpE encoding propionyl coenzyme A synthetase was placed under the control of the IPTG-inducible taclacUV5 promoter while the polyhydroxyalkanoate synthesis operon (phaBCA) from Acinetobacter sp. RA3849 was co-expressed under the control of the arabinose-inducible araBAD promoter. *S. enterica*, harboring both constructs, was grown in medium containing a fixed substrate concentration and the composition of the copolymer was varied between 2 mol% and 25 mol% 3HV by controlling the IPTG level in the medium. This ‘dial-a-composition’ system could be valuable in cases where the substrate concentration of a feedstream for P(3HB-*co*-3HV) biosynthesis is not adjustable.

As propionic acid is toxic for *R. eutropha*, alternative means of introducing 3HV monomeric unit have therefore been explored. ProCoA is an intermediate in the degradation pathway of certain amino acids, i.e., threonine, valine, and isoleucine. Hence, the introduction of these amino acid pathways into the parent *R. eutropha* strains could provide more ProCoA intracellularly, which is readily condenses with AcCoA to form the copolymer. With *R. eutropha* R3, Steinbuchel and Pieper (1992) successfully developed the biosynthesis of P(3HB-*co*-3HV) from fructose, gluconate, succinate, acetate, and lactate without the supplementation of the growth medium with propionic acid or other 3HV-yielding substrates. However, the molar 3 HV fraction is still low, only up to 7%.

Addition of threonine, isoleucine, and valine to cultures of *R. eutropha* SH-69 resulted in the incorporation of 53, 41, and 15% 3HV fraction, respectively. When the concentration of amino acid supplements exceeds 10 mM, the fraction of 3HV in the copolymer is directly related to the concentration of the amino acid.

Also, there is a US patent application confirming that the introduction of these amino acid pathways could enhance the 3HV fraction both in transgenic plant and recombinant bacteria.

CHAPTER 6
CONCLUSIONS

6.1 Conclusions

The following conclusions can be drawn out from this research.

1. There are four metabolic networks describing the metabolic behavior of *Ralstonia eutropha* NCIMB 11599. These pathways are depending upon used substrate and level of nitrogen limitation. Mainly, there are such similarities among them; namely, modified pattern of pentose-phosphate (PP) pathway, acetyl coenzyme A formation, tricarboxylic acid (TCA) cycle, 3-hydroxybutyrate formation route, ammonium assimilation via GS-GOGAT system, residual biomass formation, oxidative phosphorylation and respiratory pathway, and phosphoenolpyruvate (PEP) carboxykinase as the only route of anaplerotic pathway. As for differences, there are at the catabolism of glucose and propionic acid, 3-hydroxyvalerate formation route, and NADPH-linked malic enzyme as an additional source of NADPH production. When glucose is used as a substrate, either as a sole or mixture with propionic acid, the catabolism of glucose is accomplished by the cyclic mode of Entner-Doudoroff (ED) pathway. While in the case that propinic acid is used as a sole carbon and energy source, the ED pathway operates in the reversed direction and named as the gluconeogenesis. In addition, if propinic acid is used as a sole or mixture with glucose, it is catabolized via methylcitric acid (MCA) cycle. And with the case that propionic acid is present, an additional route for 3-hydroxyvalerate formation is included in the network. At the two extreme cases, NADPH-linked malic enzyme is active. These two extreme cases are a) the case where propionic acid is used as a sole carbon and energy source under the moderate nitrogen limitation corresponding to the molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium of 102 and 100%, respectively; and b) the case where an equimolar mixture of glucose and propionic acid under the utmost severe nitrogen limitation corresponding to the molar carbon-to-nitrogen ratio and molar fraction of propionic acid in the reaction medium of 200 and 50%, respectively.

2. From the calculation of maximum theoretical molar 3HV fraction in the copolymer, it can be concluded that the theoretical values are much more than the ones computed from the experiment data. This suggests that there is still much more room for improvement of 3HV fraction in the copolymer. Interestingly, the improvement can be systematically attained by the application of metabolic engineering.

3. According to the analysis of flux split ratio, there are two principle nodes in the metabolic network where a mixture of glucose and propionic acid is used as a carbon source. These nodes are AcCoA and ProCoA. Providing that the increased molar fraction of 3HV is desired, flux split ratios at these two nodes need to be modified. The modifications can be achieved by; a) increasing the flux of AcCoA that condenses with ProCoA to form more 3HV; b) decreasing the flux of AcCoA that condenses to form less 3HB; c) increasing the flux of ProCoA that condenses with AcCoA to form more 3HV; and d) decreasing the flux of ProCoA that enters MCA cycle.

4. From the above conclusions, the target sites for metabolic engineering are as follows; a) engineering the active site of 3-ketothiolase for much more active with ProCoA comparing to AcCoA; b) amplifying PHB synthase; c) fully or partially blocking isocitrate dehydrogenase enzyme; d) fully or partially blocking methylcitrate lyase; e) amplifying glucose-6-phosphate dehydrogenase enzyme to produce more NADPH; and f) increasing intracellular ProCoA by feeding additional ProCoA-yielding substrate and/or introducing an additional ProCoA formation route.

6.2 Recommendations

The following are the recommendations for this research.

As the proposed metabolic networks are developed based on only available information on biochemistry, much more experimental study should be conducted to confirm the presence of enzyme(s) or pathway(s). As a matter of fact, enzyme assay of the key enzymes in each metabolic pathway should be accomplished. These key enzymes include glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (GN6DH), phosphoglucoisomerase (PGI), fructose-1,6-bisphosphatase (FBP), 2-keto-3-deoxy-6-phosphogluconate aldolase or Entner-Doudoroff aldolase (EDA), methylcitric acid synthase (MCAS), isocitrate dehydrogenase (IDH), isocitrate lyase (ILY), 3-ketothiolase, acetoacetyl coenzyme A reductase, PHB polymerase, glutamine synthetase (GS), NADPH-linked glutamate synthase (GOGAT), NADPH-linked glutamate dehydrogenase (GDH), transhydrogenase (THD), phosphoenolpyruvate carboxykinase (PPC), and NADPH-linked malic enzyme (MAL).

6.3 Future work

The following are the possible work in the future that could follow this research.

1. The ultimate aim of the research might be at maximizing the productivity of P(3HB-*co*-3-HV).
2. Genetic engineering according to the suggested rational modifications should be pursued to check whether they can render much more molar fraction of 3HV experimentally.

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APPENDICES

APPENDIX A:
Evaluation of all specific measured rates

A1 Evaluation of accumulated residual biomass
A2 Evaluation of specific growth rates
A3 Evaluation of specific uptake rates of glucose, propionic acid, and nitrogen
A4 Evaluation of specific rates of 3-HB and 3-HV monomeric units

This section covers all step-by-step procedures for the evaluation of all specific measured rates necessary for metabolic flux analysis. The procedure started with collection of time-series data reported in the master thesis of Kasipar (2002). Then the data were graphically plotted and interpreted according to the corresponding expressions derived from material balance models. Finally, specific rates as well as their consistency were obtained by application of linear regression analyses

Hereby, FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was controlled, is selected as a demonstration for the evaluation of those rates. Graphical presentation is patched at the end of each section. As well, all raw data and rate calculations can be consulted from *Table A1*.

A1 Evaluation of accumulated residual biomass

- Plot the residual biomass amount in the 2nd column versus the elapsed time in the 1st column.
- Select the period of interest for further linear regression analysis, specifically, including only the growth phase and excluding the lag and dead phases.
- Over the period of interest, the corresponding expression describing the smoothed time-series data of the residual biomass can be obtained with the statistical R^2 value.
- Analytical integration of the expression at each time interval yields the accumulated residual biomass, $\int_{t_o}^{t_i} X_v(t)dt$, as shown in the 11th column.

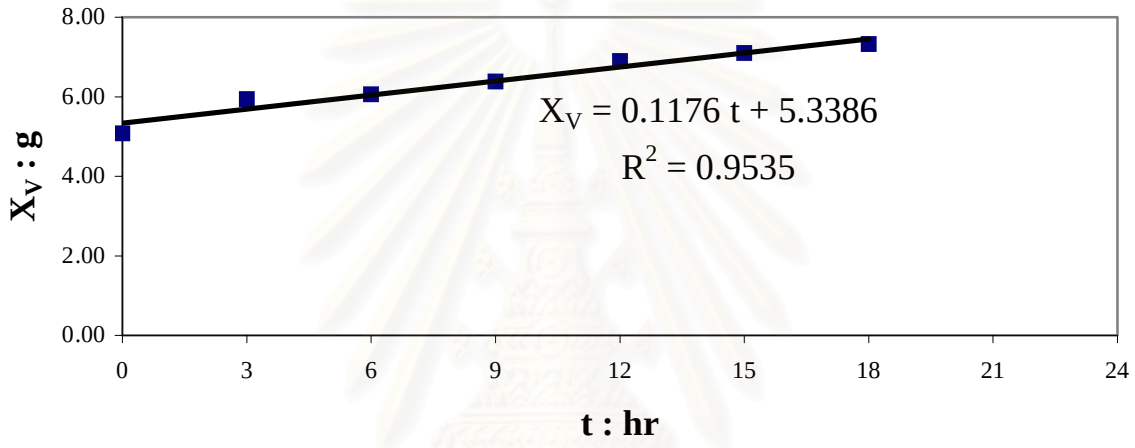

Figure A1: Evaluation of accumulated residual biomass for FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was successfully controlled.

A2 Evaluation of specific growth rates

- Recalled from the material balance of viable residual biomass in Chapter 3, the following expression can be obtained.

$$\ln X_v(t_i) \quad - \quad \ln X_v(t_o) \quad = \quad \mu[t_i - t_o]$$

- From the data in the 2nd column, its natural logarithmic value can be obtained as the 12th column.
- Plot the natural logarithmic values from the 12th column versus the elapsed time in the 1st column.
- Select the time period of interest for further linear regression analysis, specifically, the same period of time as chosen for the evaluation of the accumulated residual biomass.
- The slope of linear line over the period of interest represents the corresponding specific growth rate obtained together with the statistical R^2 value.

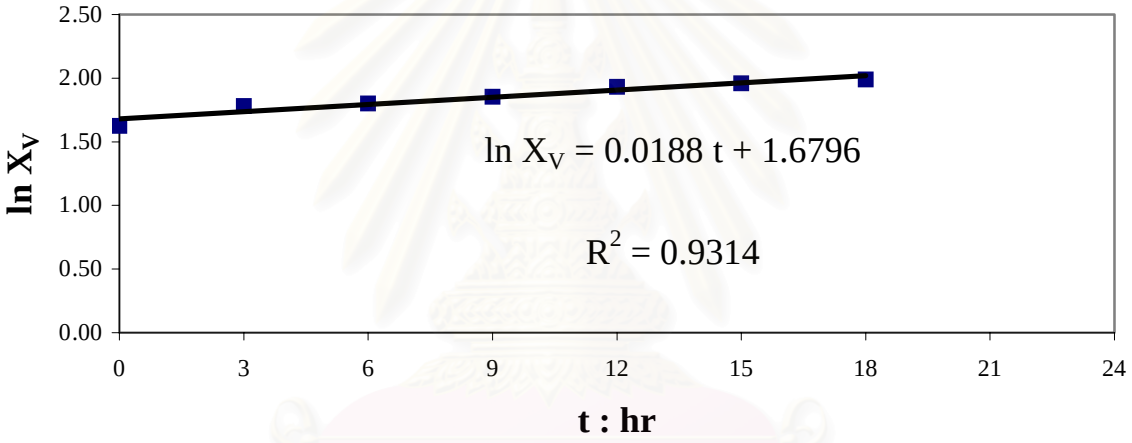

Figure A2: Evaluation of specific growth rate for FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was successfully controlled.

A3 Evaluation of specific uptake rates of glucose, propionic acid, and nitrogen

- Recalled from the material balance of substrate demonstrated in Chapter 3, the following expression can be obtained.

$$C_S(t_i) - C_S(t_o) = -r_S \int_{t_o}^{t_i} X_v(t)dt$$

- Equivalently, the following expression can be deduced from the above equation as an accumulation of the stepwise discrete data of the substrate concentration along the two consecutive points of time.

$$[C_S(t_i) - C_S(t_{i-1})] + [C_S(t_{i-1}) - C_S(t_o)] = -r_S \int_{t_o}^{t_i} X_v(t)dt$$

- The net glucose consumption during each time interval is represented in the 13th column and is calculated by subtracting the present row of the 3rd column from the previous row of the 4th column.
- Then the total accumulated glucose consumed is reported in the 14th column by summing the former accumulated consumed with the net consumed amounts.
- Plot the obtained data in the 14th column versus the accumulated residual biomass in the 11th column.
- Select the time period of interest for further linear regression analysis, specifically, the same period of time as chosen for the evaluation of the accumulated residual biomass.
- The slope of linear line over the period of interest represents the corresponding specific uptake rate of glucose obtained together with the statistical R^2 value.
- The specific uptake rates of propionic acid and nitrogen, accompanied by the corresponding R^2 values, can thus be obtained likewise.

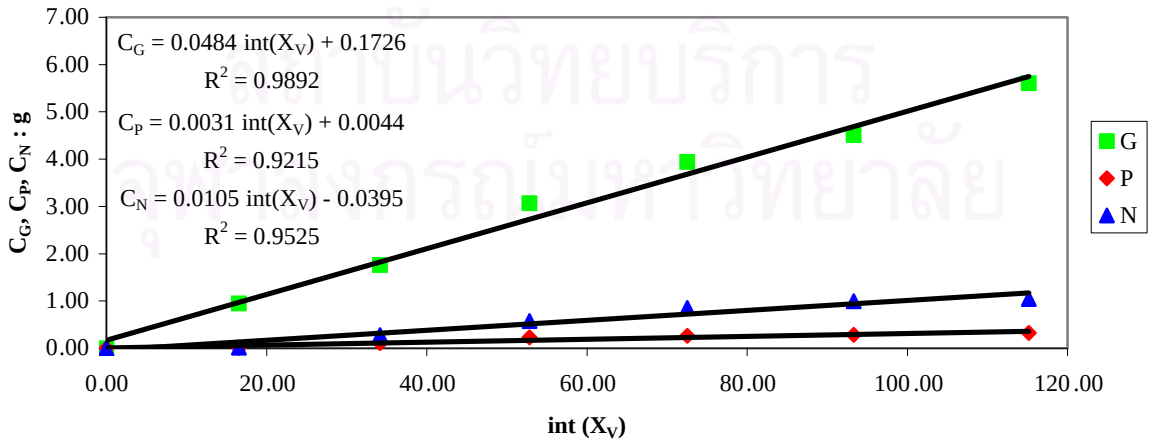

Figure A3: Evaluation of specific uptake rates of glucose, propionic acid, and nitrogen for FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was successfully controlled.

A4 Evaluation of specific formation rates of 3-HB and 3-HV monomeric units

- Recalled from the material balance of metabolic product demonstrated in Chapter 3, the following expression can be obtained.

$$C_P(t_i) - C_P(t_o) = r_P \int_{t_o}^{t_i} X_v(t)dt$$

- Plot the amount of 3-HB monomeric unit in the 9th column versus the accumulated residual biomass in the 11th column.
- Select the time period of interest for further linear regression analysis, specifically, the same period of time as chosen for the evaluation of the accumulated residual biomass.
- The slope of linear line over the period of interest represents the corresponding specific production rate of 3-HB monomeric unit obtained together with the statistical R^2 value.
- The specific formation rate of 3-HV monomeric unit, accompanied by the corresponding R^2 values, can thus be obtained likewise.

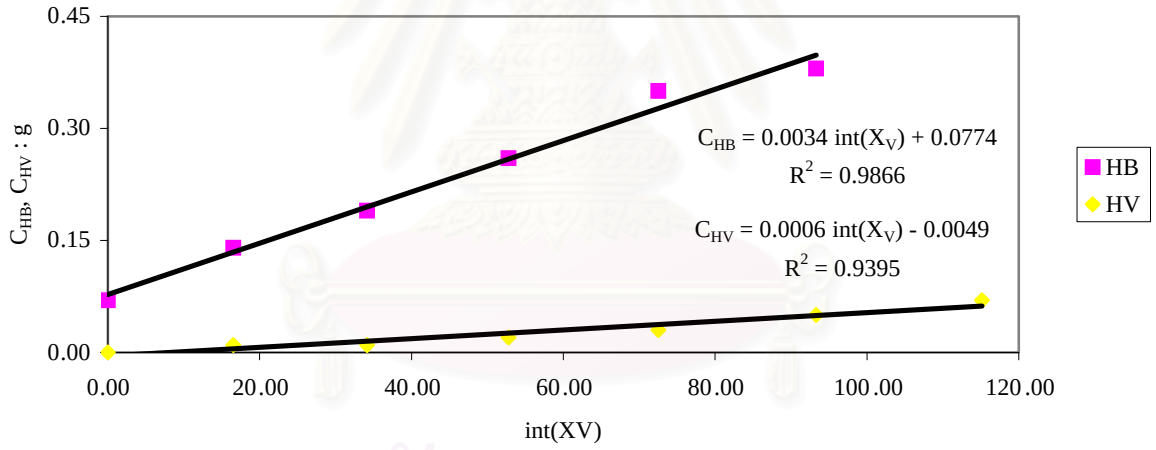

Figure A4: Evaluation of specific formation rates of 3-HB and 3-HV monomeric units for FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was successfully controlled.

Table A1: Time-series experimental and calculated data for evaluation of all specific rates of FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was controlled. The experimental data, collected from Kasipar (2002), are those in the first to tenth columns; whereas the calculated data are those in the eleventh to eighteenth columns.

t (hr) [1]	X_V (g) [2]	G (g)		P (g)		N (g)		HB (g) [9]	HV (g) [10]	int(X_V) [11]	ln X_V [12]	Consumed G (g)		Consumed P (g)		Consumed N (g)	
		Before [a] [3]	After [b] [4]	Before [a] [5]	After [b] [6]	Before [a] [7]	After [b] [8]					Net [13]	Accumulated [14]	Net [15]	Accumulated [16]	Net [17]	Accumulated [18]
0	5.08	2.14	2.41	0.19	0.19	0.43	0.43	0.07	0.00	0.00	1.63	0.00	0.00	0.00	0.00	0.00	0.00
3	5.94	1.46	2.30	0.18	0.25	0.42	0.69	0.14	0.01	16.55	1.78	0.95	0.95	0.01	0.01	0.02	0.02
6	6.06	1.49	2.47	0.16	0.28	0.43	0.47	0.19	0.01	34.15	1.80	0.81	1.76	0.10	0.11	0.26	0.28
9	6.38	1.17	2.38	0.16	0.17	0.17	0.27	0.26	0.02	52.81	1.85	1.30	3.06	0.12	0.23	0.30	0.58
12	6.90	1.51	2.26	0.14	0.21	0.00	0.15	0.35	0.03	72.53	1.93	0.87	3.94	0.03	0.26	0.27	0.85
15	7.10	1.69	2.74	0.19	0.20	0.00	0.05	0.38	0.05	93.31	1.96	0.57	4.51	0.02	0.29	0.15	1.00
18	7.32	1.64	2.58	0.17	0.20	0.00	0.31	0.32	0.07	115.15	1.99	1.10	5.61	0.04	0.32	0.05	1.04
21	6.18	1.10	2.50	0.16	0.24	0.15	0.48	0.21	0.05	135.40	1.82	1.48	7.08	0.04	0.36	0.16	1.20
24	5.06	1.02	2.40	0.19	0.09	0.20	0.30	0.16	0.02	152.26	1.62	1.48	8.57	0.05	0.41	0.28	1.48

Note: *a* indicates the amount of the substrate just before feeding the fresh reaction medium into the fermenter.
b indicates the amount of the substrate just after feeding the fresh reaction medium into the fermenter.

Table A2: Summary of all specific measured rates necessary for metabolic flux analysis.

Protocol	Natural variable		μ (h^{-1})	q_G (g G/g RB/h)	q_P (g P/g RB/h)	q_A (g A/g RB/h)	q_{HB} (g HB/g RB/h)	q_{HV} (g HV/g RB/h)	Carbon recovery (%)	q_G/q_P (mol G/mol P)	3HV molar fraction (%)
	C/N	%P									
FB01	32.7	14.7	0.0188 (0.9314)	0.0484 (0.9892)	0.0031 (0.9215)	0.0105 (0.9525)	0.0034 (0.9866)	0.0006 (0.9395)	54.06	6.42	13.18
FB02	171.3	14.7	0.0078 (0.3259)	0.0447 (0.9879)	0.0024 (0.9622)	0.0048 (0.9581)	0.0068 (0.9498)	0.0011 (0.7934)	43.05	7.66	12.21
FB03	32.7	85.4	0.0195 (0.8914)	0.0337 (0.9176)	0.0082 (0.8499)	0.0060 (0.9351)	0.0014 (0.8553)	0.0005 (0.7995)	59.77	1.69	23.50
FB04	171.3	85.4	0.0061 (0.9186)	0.0170 (0.9140)	0.0040 (0.9456)	0.0016 (0.9921)	0.0006 (0.8608)	0.0009 (0.9154)	43.48	1.75	56.33
FB05	4	50	0.0086 (0.9892)	0.0519 (0.9886)	0.0035 (0.9764)	0.0628 (0.9969)	0.0023 (0.9524)	0.0008 (0.8714)	26.23	6.10	23.03
FB06	200	50	0.0103 (0.8175)	0.0228 (0.9397)	0.0026 (0.8837)	0.0042 (0.9129)	0.0064 (0.9799)	0.0019 (0.9149)	92.98	3.61	20.34
FB07	102	0	0.0144 (0.9553)	0.0547 (0.9757)	ND	0.0059 (0.8929)	0.0179 (0.7693)	ND	77.25		0.00
FB08	102	100	0.0044 (0.8697)	ND	0.0132 (0.8041)	0.0033 (0.9347)	0.0034 (0.8894)	0.0021 (0.8966)	82.06		34.69
FB09	102	50	0.0114 (0.9314)	0.0488 (0.9577)	0.0069 (0.7010)	0.0050 (0.9725)	0.0035 (0.8079)	0.0019 (0.9093)	37.44	2.91	31.83
FB10	102	50	0.0147 (0.6002)	0.0300 (0.9719)	0.0039 (0.9458)	0.0062 (0.9591)	0.0040 (0.8319)	0.0026 (0.8586)	78.06	3.16	35.86
FB11	102	50	0.0176 (0.9161)	0.0330 (0.9557)	0.0063 (0.9008)	0.0056 (0.9168)	0.0039 (0.9652)	0.0023 (0.9244)	73.81	2.15	33.65
FB12	102	50	0.0160 (0.9782)	0.0572 (0.9499)	0.0075 (0.7119)	0.0026 (0.8656)	0.0018 (0.8484)	0.0019 (0.9295)	37.03	3.14	47.58
FB13	102	50	0.0162 (0.5926)	0.0216 (0.8295)	0.0066 (0.9403)	0.0075 (0.9341)	0.0018 (0.8140)	0.0008 (0.8316)	78.14	1.35	27.65

Note: ND means the value is non-detectable.

APPENDIX B:

Metabolite accumulation rate vectors and metabolic reactions

B1. Metabolite accumulation rate vectors
B2. Metabolic reactions

B1. Metabolite accumulation rate vectors

GLU	Glucose
G6P	Glucose-6-phosphate
GN6P	6-phosphogluconate or gluconate-6-phosphate
GA3P	Glyceraldehyde-3-phosphate
F6P	Fructose-6-phosphate
G3P	Glycerate-3-phosphate or 3-phosphoglycerate
PEP	Phosphoenolpyruvate
PYR	Pyruvate
R5P	Ribulose-5-phosphate
E4P	Erythrose-4-phosphate
PRO	Propionic acid
AcCoA	Acetyl coenzyme A
ProCoA	Propionyl coenzyme A
OAA	Oxaloacetate
IsoCit	Isocitrate
AKG	α-ketoglutarate
SUC	Succinate
MAL	Malate
MetCit	2-methylcitrate
AcAcCoA	Acetoacetyl coenzyme A
KetVaCoA	3-ketovaleryl coenzyme A
HB	3-hydroxybutyrate monomeric unit
HV	3-hydroxyvalerate monomeric unit
GLUT	L-glutamate
GLUM	L-glutamine
NH_3	Ammonium
RB	Residual biomass
CO_2	Carbondioxide
O_2	Oxygen
ADP	Adenosine 5-diphosphate
ATP	Adenosine 5-triphosphate
FAD	Flavin adenine dinucleotide, oxidized form
$FADH_2$	Flavin adenine dinucleotide, reduced form
NAD	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form

B2. Metabolic reactions

Cyclic mode of Entner-Doudoroff pathway (ED)

1) GLU + ATP = G6P + ADP
2) G6P + NADP = GN6P + NADPH
3) GN6P = GA3P + PYR
4) GA3P + NAD + ADP = G3P + NADH + ATP
5) G3P = PEP
6) PEP + ADP = PYR + ATP
7) 2 GA3P = F6P
8) F6P = G6P

Modified pattern of pentose-phosphate pathway (PP)

9) GN6P + H_2O + 2 NADP = R5P + CO_2 + 2 NADPH
10) F6P + GA3P = R5P + E4P

2-Methylcitric acid cycle (MCA)

11) PRO + ATP + CoA = ProCoA + ADP
12) ProCoA + OAA = MetIsoCit + CoA
13) MetIsoCit = PYR + SUC

Acetyl-CoA formation

14) PYR + CoA + NAD = AcCoA + CO_2 + NADH
15) ProCoA = AcCoA + CO_2

Tricarboxylic acid cycle (TCA)

16) AcCoA + OAA + H_2O = IsoCit + CoA
17) IsoCit + NADP = AKG + NADPH + CO_2
18) AKG + NAD + ADP = SUC + CO_2 + NADH + ATP
19) SUC + H_2O + FAD = MAL + $FADH_2$
20) MAL + NAD = OAA + NADH

PHA formation

21) 2 AcCoA = AcAcCoA + CoA
22) AcAcCoA + NADPH = PHB + NADP + CoA
23) AcCoA + ProCoA = KetVaCoA + CoA
24) KetVaCoA + NADPH = PHV + NADP + CoA

GS-GOGAT system

25) AKG + GLUM + NADPH = 2 GLUT + NADP
26) GLUT + NH_3 + ATP = GLUM + ADP

Residual biomass formation

(Assume : MW of Biomass = 100; $C_4H_{6.9}O_{1.64}N_{0.98}$; 5% Ash)

27) 0.021 G6P + 0.007 F6P + 0.09 R5P + 0.036 E4P
+ 0.013 GA3P + 0.15 G3P + 0.052 PEP + 0.283 PYR
+ 0.374 AcCoA + 0.179 OAA + 0.832 GLUT + 0.025 GLUM
+ 4.11ATP + 0.826 NADPH + 0.312 NAD
= RB + 0.751 AKG + 0.261 CO_2
+ 4.11 ADP + 0.826 NADP + 0.312 NADH

Oxidative phosphorylation (P/O = 2)

28) 2 NADH + O_2 + 4 ADP = 2 H_2O + 4 ATP + 2 NAD

29) 2 FADH + O_2 + 2 ADP = 2 H_2O + 2 ATP + 2 FAD

Futile cycle

30) ATP = ADP

Transport

31) $NH_{3\ (ext.)}$ = $NH_{3\ (int)}$

32) $CO_{2\ (int)}$ = $CO_{2\ (ext)}$

33) $O_{2\ (ext)}$ = $O_{2\ (int)}$

Transhydrogenation

34) NADPH + NAD = NADH + NADP

Anaplerotic reactions

35) PEP + CO_2 = OAA

36) PYR + CO_2 = OAA

APPENDIX C:

Metabolic pathway analysis

Table C1: All possible metabolic networks for the aerobic cultivation of *Ralstonia eutropha* NCIMB 11599 growing on glucose as a sole carbon and energy source.

Case	Flux inclusion					Rank(G_c)*	Feasibility
	V_7	V_8	V_{34}	V_{35}	V_{36}		
A1	+	+	+	+	+	N	F
A2	+	+	+	+	-	N	F
A3	+	+	+	-	+	N	I
A4	+	+	+	-	-	N	I
A5	+	+	-	+	+	N	F
A6	+	+	-	+	-	FR	F
A7	+	+	-	-	+	FR	I
A8	+	+	-	-	-	FR	I
A9	+	-	+	+	+	N	F
A10	+	-	+	+	-	FR	F
A11	+	-	+	-	+	FR	I
A12	+	-	+	-	-	FR	I
A13	+	-	-	+	+	FR	F
A14	+	-	-	+	-	FR	F
A15	+	-	-	-	+	FR	I
A16	+	-	-	-	-	FR	I
A17	-	+	+	+	+	N	F
A18	-	+	+	+	-	FR	F
A19	-	+	+	-	+	FR	I
A20	-	+	+	-	-	FR	I
A21	-	+	-	+	+	FR	F
A22	-	+	-	+	-	FR	F
A23	-	+	-	-	+	FR	I
A24	-	+	-	-	-	FR	I
A25	-	-	+	+	+	N	I
A26	-	-	+	+	-	FR	I
A27	-	-	+	-	+	FR	I
A28	-	-	+	-	-	FR	I
A29	-	-	-	+	+	FR	I
A30	-	-	-	+	-	FR	I
A31	-	-	-	-	+	FR	I
A32	-	-	-	-	-	FR	I

Note: + indicates that the flux is included in the metabolic network.
- indicates that the flux is excluded in the metabolic network.
FR means the stoichiometric matrix of that case is of full rank.
N means the stoichiometric matrix of that case is not of full rank.
F means the metabolic network of that case is biochemically-feasible.
I means the metabolic network of that case is biochemically-infeasible.
Highlighted are the metabolic network cases that qualify for both biochemical and mathematical feasibility.

Table C2: All possible metabolic networks for the aerobic cultivation of *Ralstonia eutropha* NCIMB 11599 growing on propionic acid as a sole carbon and energy source.

Case	Flux inclusion				Rank(G_c)*	Feasibility
	V_{15}	V_{34}	V_{35}	V_{36}		
B1	+	+	+	+	N	I
B2	+	+	+	-	N	I
B3	+	+	-	+	N	I
B4	+	+	-	-	N	I
B5	+	-	+	+	N	I
B6	+	-	+	-	N	I
B7	+	-	-	+	N	I
B8	+	-	-	-	N	I
B9	-	+	+	+	N	F
B10	-	+	+	-	FR	F
B11	-	+	-	+	FR	I
B12	-	+	-	-	FR	I
B13	-	-	+	+	FR	F
B14	-	-	+	-	FR	F
B15	-	-	-	+	FR	I
B16	-	-	-	-	FR	I

Note: + indicates that the flux is included in the metabolic network.
- indicates that the flux is excluded in the metabolic network.
FR means the stoichiometric matrix of that case is of full rank.
N means the stoichiometric matrix of that case is not of full rank.
F means the metabolic network of that case is biochemically-feasible.
I means the metabolic network of that case is biochemically-infeasible.
Highlighted are the metabolic network cases that qualify for both biochemical and mathematical feasibility.

Table C3: All possible metabolic networks for the aerobic cultivation of *Ralstonia eutropha* NCIMB 11599 growing on a mixture of glucose and propionic acid.

Case	Flux inclusion						Rank(G_c)*	Feasibility
	V_7	V_8	V_{15}	V_{34}	V_{35}	V_{36}		
C1	+	+	+	+	+	+	N	I
C2	+	+	+	+	+	-	N	I
C3	+	+	+	+	-	+	N	I
C4	+	+	+	+	-	-	N	I
C5	+	+	+	-	+	+	N	I
C6	+	+	+	-	+	-	N	I
C7	+	+	+	-	-	+	N	I
C8	+	+	+	-	-	-	N	I
C9	+	+	-	+	+	+	N	F
C10	+	+	-	+	+	-	N	F
C11	+	+	-	+	-	+	N	I
C12	+	+	-	+	-	-	N	I
C13	+	+	-	-	+	+	N	F
C14	+	+	-	-	+	-	FR	F
C15	+	+	-	-	-	+	FR	I
C16	+	+	-	-	-	-	FR	I
C17	+	-	+	+	+	+	N	I
C18	+	-	+	+	+	-	N	I
C19	+	-	+	+	-	+	N	I
C20	+	-	+	+	-	-	N	I
C21	+	-	+	-	+	+	N	I
C22	+	-	+	-	+	-	N	I
C23	+	-	+	-	-	+	N	I
C24	+	-	+	-	-	-	N	I
C25	+	-	-	+	+	+	N	F
C26	+	-	-	+	+	-	FR	F
C27	+	-	-	+	-	+	FR	I
C28	+	-	-	+	-	-	FR	I
C29	+	-	-	-	+	+	FR	F
C30	+	-	-	-	+	-	FR	F
C31	+	-	-	-	-	+	FR	I
C32	+	-	-	-	-	-	FR	I
C33	-	+	+	+	+	+	N	I
C34	-	+	+	+	+	-	N	I
C35	-	+	+	+	-	+	N	I
C36	-	+	+	+	-	-	N	I
C37	-	+	+	-	+	+	N	I
C38	-	+	+	-	+	-	N	I
C39	-	+	+	-	-	+	N	I
C40	-	+	+	-	-	-	N	I

Table C3 (cont.): All possible metabolic networks for the aerobic cultivation of *Ralstonia eutropha* NCIMB 11599 growing on a mixture of glucose and propionic acid.

Case	Flux inclusion						Rank(G_c)*	Feasibility
	V_7	V_8	V_{15}	V_{34}	V_{35}	V_{36}		
C41	-	+	-	+	+	+	N	I
C42	-	+	-	+	+	-	FR	I
C43	-	+	-	+	-	+	FR	I
C44	-	+	-	+	-	-	FR	I
C45	-	+	-	-	+	+	FR	I
C46	-	+	-	-	+	-	FR	I
C47	-	+	-	-	-	+	FR	I
C48	-	+	-	-	-	-	FR	I
C49	-	-	+	+	+	+	N	I
C50	-	-	+	+	+	-	N	I
C51	-	-	+	+	-	+	N	I
C52	-	-	+	+	-	-	N	I
C53	-	-	+	-	+	+	N	I
C54	-	-	+	-	+	-	N	I
C55	-	-	+	-	-	+	N	I
C56	-	-	+	-	-	-	N	I
C57	-	-	-	+	+	+	N	I
C58	-	-	-	+	+	-	FR	I
C59	-	-	-	+	-	+	FR	I
C60	-	-	-	+	-	-	FR	I
C61	-	-	-	-	+	+	FR	I
C62	-	-	-	-	+	-	FR	I
C63	-	-	-	-	-	+	FR	I
C64	-	-	-	-	-	-	FR	I

Note: + indicates that the flux is included in the metabolic network.
- indicates that the flux is excluded in the metabolic network.
FR means the stoichiometric matrix of that case is of full rank.
N means the stoichiometric matrix of that case is not of full rank.
F means the metabolic network of that case is biochemically-feasible.
I means the metabolic network of that case is biochemically-infeasible.
Highlighted are the metabolic network cases that qualify for both biochemical and mathematical feasibility.

Table C4: Condition number of metabolic network case A of which the linear algebraic system is of full rank and biochemically-feasible.

Case	Condition number
A6	669.83
A10	568.98
A13	623.56
A14	250.65
A18	564.52
A21	622.07
A22	239.00

Table C5: Condition number of metabolic network case B of which the linear algebraic system is of full rank and biochemically-feasible.

Case	Condition number
B10	619.35
B13	668.41
B14	378.92

Table C6: Condition number of metabolic network case C of which the linear algebraic system is of full rank and biochemically-feasible.

Case	Condition number
C14	756.94
C26	641.94
C29	704.67
C30	264.72

Table C7: Calculated flux sensitivities with respect to changes in measured fluxes in metabolic network case A13 where *Ralstonia eutropha* NCIMB 11599 is growing on glucose as a sole carbon and energy source.

Flux	Glucose uptake rate	3HB formation rate	RB formation rate	Nitrogen uptake rate
V2	0.74	0.26	0.12	0.09
V3	0.66	0.34	0.12	0.11
V4	0.14	0.86	0.35	0.29
V5	0.06	0.94	0.25	0.31
V6	-0.63	1.63	0.74	0.04
V7	0.17	-0.17	-0.09	-0.06
V9	-0.09	0.09	0.10	0.03
V10	0.09	-0.09	-0.01	-0.03
V14	-0.06	2.06	0.62	0.19
V16	-0.23	0.23	0.72	0.24
V17	-0.40	0.40	0.82	0.30
V18	-0.57	0.57	1.25	-0.14
V19	-0.66	0.66	1.30	-0.11
V20	-0.74	0.74	1.34	-0.09
V21	0.09	0.91	-0.17	-0.03
V25	0.09	-0.09	0.37	0.47
V26	0.09	-0.09	0.10	0.80
V28	-0.61	2.11	1.94	0.12
V29	-0.33	0.33	0.65	-0.06
V30	-5.26	12.26	7.16	-0.25
V32	-1.71	3.71	3.54	0.07
V33	-0.94	2.44	2.58	0.06
V35	0.60	-0.60	-0.50	0.30
Accumulated	14.94	31.57	24.83	4.20

Table C8: Calculated flux sensitivities with respect to changes in measured fluxes in metabolic network case B10 where *Ralstonia eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source.

Flux	Propionic acid uptake rate	3HB formation rate	3HV formation rate	RB formation rate	Nitrogen uptake rate
V2	0.00	0.00	0.00	0.05	0.00
V4	0.00	0.00	0.00	-0.21	0.00
V5	0.00	0.00	0.00	-0.36	0.00
V6	0.00	0.00	0.00	-0.26	-0.50
V7	0.00	0.00	0.00	0.08	0.00
V8	0.00	0.00	0.00	0.08	0.00
V9	0.00	0.00	0.00	0.05	0.00
v10	0.00	0.00	0.00	0.04	0.00
v12	1.00	0.00	-1.00	0.15	0.00
v13	1.00	0.00	-1.00	0.15	0.00
v14	1.00	0.00	-1.00	-0.39	-0.50
v16	1.00	-2.00	-2.00	-0.47	-0.50
v17	1.00	-2.00	-2.00	-0.47	-0.50
v18	1.00	-2.00	-2.00	-0.13	-1.00
v19	2.00	-2.00	-3.00	0.02	-1.00
v20	2.00	-2.00	-3.00	0.02	-1.00
v21	0.00	1.00	0.00	-0.07	0.00
v23	0.00	0.00	1.00	-0.07	0.00
v25	0.00	0.00	0.00	0.42	0.50
v26	0.00	0.00	0.00	0.15	0.83
v28	2.50	-3.50	-4.50	-0.98	-1.75
v29	1.00	-1.00	-1.50	0.01	-0.50
v30	12.00	-18.00	-23.00	-8.75	-10.33
v32	3.00	-4.00	-5.00	-0.52	-2.50
v33	3.50	-4.50	-6.00	-0.97	-2.25
v34	1.00	-3.00	-3.00	-1.55	-1.00
v35	0.00	0.00	0.00	-0.16	0.50
Accumulated	33.00	45.00	59.00	16.58	25.17

Table C9: Calculated flux sensitivities with respect to changes in measured fluxes in metabolic network case B13 where *Ralstonia eutropha* NCIMB 11599 is growing on propionic acid as a sole carbon and energy source.

Flux	Propionic acid uptake rate	3HB formation rate	3HV formation rate	RB formation rate	Nitrogen uptake rate
V2	0.00	0.00	0.00	0.05	0.00
V4	0.00	0.00	0.00	-0.21	0.00
V5	0.00	0.00	0.00	-0.36	0.00
V6	1.00	-3.00	-3.00	-1.81	-1.50
V7	0.00	0.00	0.00	0.08	0.00
V8	0.00	0.00	0.00	0.08	0.00
V9	0.00	0.00	0.00	0.05	0.00
v10	0.00	0.00	0.00	0.04	0.00
v12	1.00	0.00	-1.00	0.15	0.00
v13	1.00	0.00	-1.00	0.15	0.00
v14	1.00	0.00	-1.00	-0.39	-0.50
v16	1.00	-2.00	-2.00	-0.47	-0.50
v17	1.00	-2.00	-2.00	-0.47	-0.50
v18	1.00	-2.00	-2.00	-0.13	-1.00
v19	2.00	-2.00	-3.00	0.02	-1.00
v20	3.00	-5.00	-6.00	-1.53	-2.00
v21	0.00	1.00	0.00	-0.07	0.00
v23	0.00	0.00	1.00	-0.07	0.00
v25	0.00	0.00	0.00	0.42	0.50
v26	0.00	0.00	0.00	0.15	0.83
v28	2.50	-3.50	-4.50	-0.98	-1.75
v29	1.00	-1.00	-1.50	0.01	-0.50
v30	13.00	-21.00	-26.00	-10.30	-11.33
v32	3.00	-4.00	-5.00	-0.52	-2.50
v33	3.50	-4.50	-6.00	-0.97	-2.25
v35	-1.00	3.00	3.00	1.39	1.50
v36	-1	3	3	1.5472	1
Accumulated	37.00	57.00	71.00	22.42	29.17

Table C10: Calculated flux sensitivities with respect to changes in measured fluxes in metabolic network case C30 where *Ralstonia eutropha* NCIMB 11599 is growing on a mixture of glucose and propionic acid.

Flux	Glucose uptake rate	Propionic acid uptake rate	3HB formation rate	3HV formation rate	RB formation rate	Nitrogen uptake rate
v2	0.77	-0.08	0.23	0.23	0.10	0.08
v3	0.70	-0.10	0.30	0.30	0.09	0.10
v4	0.25	-0.25	0.75	0.75	0.27	0.25
v5	0.17	-0.28	0.83	0.83	0.16	0.28
v6	-0.58	-0.53	1.58	1.58	0.67	0.03
v7	0.15	0.05	-0.15	-0.15	-0.07	-0.05
v9	-0.08	-0.03	0.08	0.08	0.09	0.03
v10	0.08	0.03	-0.08	-0.08	0.00	-0.03
v12	-0.05	0.98	0.05	-0.95	0.17	0.02
v13	-0.20	0.93	0.20	-0.80	0.26	0.07
v14	-0.16	0.28	2.16	1.16	0.77	0.22
v16	-0.32	0.23	0.32	0.32	0.79	0.27
v17	-0.47	0.18	0.47	0.47	0.87	0.32
v18	-0.62	0.13	0.62	0.62	1.29	-0.13
v19	-0.89	1.04	0.89	-0.11	1.58	-0.04
v20	-0.97	1.01	0.97	-0.03	1.62	-0.01
v21	0.06	0.02	0.94	-0.06	-0.11	-0.02
v23	0.06	0.02	-0.06	0.94	-0.11	-0.02
v25	0.08	0.03	-0.08	-0.08	0.38	0.47
v26	0.08	0.03	-0.08	-0.08	0.11	0.81
v28	-0.75	0.58	2.25	1.25	2.13	0.17
v29	-0.45	0.52	0.45	-0.05	0.79	-0.02
v30	-5.93	1.69	12.93	7.93	8.12	-0.02
v32	-2.01	0.33	4.01	3.01	3.81	0.17
v33	-1.20	1.10	2.70	1.20	2.92	0.15
v35	0.68	0.23	-0.68	-0.68	-0.52	0.27
Accumulated	17.74	10.64	33.84	23.73	27.81	4.02

Table C11: Calculated flux sensitivities with respect to changes in measured fluxes in metabolic network case C29 where *Ralstonia eutropha* NCIMB 11599 is growing on a mixture of glucose and propionic acid.

Flux	Glucose uptake rate	Propionic acid uptake rate	3HB formation rate	3HV formation rate	RB formation rate	Nitrogen uptake rate
v2	1.00	0.00	0.00	0.00	-0.02	0.00
v3	1.00	0.00	0.00	0.00	-0.08	0.00
v4	1.00	0.00	0.00	0.00	-0.14	0.00
v5	1.00	0.00	0.00	0.00	-0.29	0.00
v6	4.00	1.00	-3.00	-3.00	-1.81	-1.50
v7	0.00	0.00	0.00	0.00	0.01	0.00
v9	0.00	0.00	0.00	0.00	0.05	0.00
v10	0.00	0.00	0.00	0.00	0.04	0.00
v12	0.00	1.00	0.00	-1.00	0.15	0.00
v13	0.00	1.00	0.00	-1.00	0.15	0.00
v14	2.00	1.00	0.00	-1.00	-0.39	-0.50
v16	2.00	1.00	-2.00	-2.00	-0.47	-0.50
v17	2.00	1.00	-2.00	-2.00	-0.47	-0.50
v18	2.00	1.00	-2.00	-2.00	-0.13	-1.00
v19	2.00	2.00	-2.00	-3.00	0.02	-1.00
v20	5.00	3.00	-5.00	-6.00	-1.60	-2.00
v21	0.00	0.00	1.00	0.00	-0.07	0.00
v23	0.00	0.00	0.00	1.00	-0.07	0.00
v25	0.00	0.00	0.00	0.00	0.42	0.50
v26	0.00	0.00	0.00	0.00	0.15	0.83
v28	5.00	2.50	-3.50	-4.50	-0.98	-1.75
v29	1.00	1.00	-1.00	-1.50	0.01	-0.50
v30	28.00	13.00	-21.00	-26.00	-10.23	-11.33
v32	6.00	3.00	-4.00	-5.00	-0.52	-2.50
v33	6.00	3.50	-4.50	-6.00	-0.97	-2.25
v35	-3.00	-1.00	3.00	3.00	1.47	1.50
v36	-3.00	-1.00	3.00	3.00	1.62	1.00
Accumulated	75.00	37.00	57.00	71.00	22.31	29.17

APPENDIX D:

Estimated flux distributions of all fermentation protocols

Table D1: Flux distribution of qualified metabolic network cases for FB01, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 14.7% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	86.52	86.52	86.52	87.27
v11	13.48	13.48	13.48	12.73
v22	12.72	12.72	12.72	18.45
v24	1.93	1.93	1.93	6.96
v27	55.94	55.94	55.94	62.10
v31	28.98	28.98	28.98	32.59
v2	-24.01	85.34	85.34	81.07
v3	-27.03	82.32	82.32	76.09
v4	188.16	78.80	78.80	62.37
v5	179.77	70.41	70.41	51.42
v6	171.09	61.73	171.09	25.26
v7	-108.96	0.39	0.39	3.71
v8	-109.36			
v9	3.02	3.02	3.02	1.72
v10	2.02	2.02	2.02	3.87
v12	19.92	19.92	19.92	14.08
v13	19.92	19.92	19.92	10.81
v14	148.15	148.15	148.15	92.95
v15				
v16	116.59	116.59	116.59	40.85
v17	116.59	116.59	116.59	37.59
v18	120.83	120.83	120.83	38.82
v19	140.75	140.75	140.75	48.00
v20	140.75	140.75	250.11	46.37
v21	8.54	8.54	8.54	15.12
v23	-2.25	-2.25	-2.25	3.61
v25	37.77	37.77	37.77	43.76
v26	32.38	32.38	32.38	37.93
v28	307.67	307.67	307.67	129.95
v29	70.38	70.38	70.38	24.00
v30	1489.25	1270.53	1379.89	301.11
v32	397.42	397.42	397.42	165.98
v33	378.05	378.05	378.05	153.95
v34		109.36		
v35	5.77	5.77	-103.59	21.31
v36			-109.36	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D2: Flux distribution of qualified metabolic network cases for FB02, representing the fermentation protocol in which C/N ratio of 171.3 and %P of 14.7% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	88.45	88.45	88.45	89.46
v11	11.55	11.55	11.55	10.54
v22	28.16	28.16	28.16	35.90
v24	3.92	3.92	3.92	9.83
v27	25.82	25.82	25.82	30.64
v31	14.36	14.36	14.36	17.40
v2	-36.51	87.91	87.91	83.19
v3	-37.90	86.51	86.51	79.66
v4	209.30	84.88	84.88	66.48
v5	205.43	81.01	81.01	60.02
v6	200.94	76.52	200.94	35.75
v7	-124.23	0.18	0.18	3.97
v8	-124.41			
v9	1.40	1.40	1.40	-0.22
v10	0.93	0.93	0.93	2.98
v12	11.50	11.50	11.50	4.16
v13	11.50	11.50	11.50	0.42
v14	167.22	167.22	167.22	105.29
v15				
v16	105.05	105.05	105.05	17.23
v17	105.05	105.05	105.05	13.48
v18	106.52	106.52	106.52	11.30
v19	118.02	118.02	118.02	9.84
v20	118.02	118.02	242.43	7.96
v21	26.23	26.23	26.23	35.11
v23	1.99	1.99	1.99	9.04
v25	17.92	17.92	17.92	23.32
v26	15.76	15.76	15.76	20.88
v28	304.56	304.56	304.56	100.29
v29	59.00	59.00	59.00	4.92
v30	1631.12	1382.28	1506.70	277.75
v32	383.77	383.77	383.77	117.05
v33	363.57	363.57	363.57	105.21
v34		124.41		
v35	3.15	3.15	-121.26	20.79
v36			-124.41	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D3: Flux distribution of qualified metabolic network cases for FB03, representing the fermentation protocol in which C/N ratio of 32.7 and %P of 85.4% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	62.82	62.82	62.82	62.19
v11	37.18	37.18	37.18	37.81
v22	5.47	5.47	5.47	9.63
v24	1.68	1.68	1.68	5.65
v27	59.12	59.12	59.12	64.18
v31	20.52	20.52	20.52	22.95
v2	-26.20	61.57	61.57	56.96
v3	-29.40	58.38	58.38	52.20
v4	142.44	54.66	54.66	40.39
v5	133.57	45.79	45.79	29.47
v6	129.46	41.68	129.46	11.73
v7	-87.37	0.41	0.41	3.04
v8	-87.78			
v9	3.19	3.19	3.19	2.17
v10	2.13	2.13	2.13	3.61
v12	44.35	44.35	44.35	40.99
v13	44.35	44.35	44.35	38.40
v14	127.68	127.68	127.68	82.87
v15				
v16	110.65	110.65	110.65	50.33
v17	110.65	110.65	110.65	47.74
v18	120.20	120.20	120.20	55.18
v19	164.55	164.55	164.55	92.28
v20	164.55	164.55	252.33	90.99
v21	1.04	1.04	1.04	5.86
v23	-2.75	-2.75	-2.75	1.88
v25	34.86	34.86	34.86	39.46
v26	25.79	25.79	25.79	29.85
v28	286.65	286.65	286.65	144.72
v29	82.27	82.27	82.27	46.14
v30	1334.46	1158.90	1246.68	384.88
v32	376.12	376.12	376.12	191.60
v33	368.92	368.92	368.92	190.87
v34		87.78		
v35	1.04	1.04	-86.75	13.11
v36			-87.78	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D4: Flux distribution of qualified metabolic network cases for FB04, representing the fermentation protocol in which C/N ratio of 171.3 and %P of 85.4% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	63.60	63.60	63.60	62.75
v11	36.40	36.40	36.40	37.25
v22	4.70	4.70	4.70	10.62
v24	6.06	6.06	6.06	12.08
v27	36.96	36.96	36.96	42.56
v31	11.65	11.65	11.65	14.47
v2	-60.48	62.83	62.83	56.29
v3	-62.48	60.83	60.83	52.13
v4	181.81	58.50	58.50	38.31
v5	176.26	52.95	52.95	30.07
v6	174.28	50.97	174.28	8.70
v7	-123.05	0.26	0.26	4.01
v8	-123.31			
v9	2.00	2.00	2.00	0.44
v10	1.33	1.33	1.33	3.39
v12	35.87	35.87	35.87	30.42
v13	35.87	35.87	35.87	26.71
v14	137.21	137.21	137.21	73.63
v15				
v16	118.98	118.98	118.98	33.05
v17	118.98	118.98	118.98	29.33
v18	125.54	125.54	125.54	32.64
v19	161.41	161.41	161.41	57.49
v20	161.41	161.41	284.72	55.63
v21	1.93	1.93	1.93	8.92
v23	3.29	3.29	3.29	10.38
v25	21.20	21.20	21.20	26.80
v26	15.14	15.14	15.14	20.17
v28	308.76	308.76	308.76	106.74
v29	80.71	80.71	80.71	28.74
v30	1610.99	1364.38	1487.69	269.04
v32	393.33	393.33	393.33	129.85
v33	389.46	389.46	389.46	135.49
v34		123.31		
v35	0.06	0.06	-123.25	17.31
v36			-123.31	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D5: Flux distribution of qualified metabolic network cases for FB05, representing the fermentation protocol in which C/N ratio of 4 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	85.91	85.91	85.91	86.24
v11	14.09	14.09	14.09	13.76
v22	7.97	7.97	7.97	10.49
v24	2.38	2.38	2.38	4.74
v27	38.18	38.18	38.18	40.49
v31	126.31	126.31	126.31	130.93
v2	32.59	85.11	85.11	83.11
v3	30.52	83.05	83.05	80.16
v4	133.17	80.64	80.64	73.07
v5	127.44	74.91	74.91	66.23
v6	68.26	15.73	68.26	-2.61
v7	-52.26	0.27	0.27	1.80
v8	-52.53			
v9	2.06	2.06	2.06	1.43
v10	1.37	1.37	1.37	2.21
v12	17.42	17.42	17.42	14.63
v13	17.42	17.42	17.42	13.12
v14	105.40	105.40	105.40	78.45
v15				
v16	84.22	84.22	84.22	48.04
v17	84.22	84.22	84.22	46.53
v18	33.85	33.85	33.85	-6.89
v19	51.27	51.27	51.27	5.47
v20	51.27	51.27	103.80	4.71
v21	5.11	5.11	5.11	8.06
v23	-0.47	-0.47	-0.47	2.31
v25	79.04	79.04	79.04	83.07
v26	110.88	110.88	110.88	115.83
v28	167.79	167.79	167.79	80.98
v29	25.64	25.64	25.64	2.73
v30	589.94	484.89	537.41	10.70
v32	178.28	178.28	178.28	64.11
v33	193.43	193.43	193.43	83.71
v34		52.53		
v35	57.20	57.20	4.67	65.98
v36			-52.53	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D6: Flux distribution of qualified metabolic network cases for FB06, representing the fermentation protocol in which C/N ratio of 200 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	78.28	78.28	78.28	78.22
v11	21.72	21.72	21.72	21.78
v22	45.99	45.99	45.99	43.67
v24	11.74	11.74	11.74	10.07
v27	58.06	58.06	58.06	56.20
v31	24.17	24.17	24.17	23.20
v2	112.06	77.06	77.06	78.49
v3	108.92	73.93	73.93	75.94
v4	35.27	70.28	70.28	75.28
v5	26.57	61.56	61.56	67.33
v6	20.52	55.52	20.52	66.39
v7	35.40	0.41	0.41	-0.57
v8	34.99			
v9	3.13	3.13	3.13	3.52
v10	2.09	2.09	2.09	1.54
v12	18.66	18.66	18.66	20.40
v13	18.66	18.66	18.66	21.36
v14	131.67	131.67	131.67	148.26
v15				
v16	23.60	23.60	23.60	47.70
v17	23.60	23.60	23.60	48.66
v18	30.96	30.96	30.96	56.86
v19	49.62	49.62	49.62	78.70
v20	49.62	49.62	14.63	79.18
v21	41.65	41.65	41.65	39.07
v23	7.40	7.40	7.40	5.48
v25	36.24	36.24	36.24	34.50
v26	28.68	28.68	28.68	27.12
v28	132.82	132.82	132.82	188.56
v29	24.81	24.81	24.81	39.35
v30	300.40	370.39	335.39	673.33
v32	201.50	201.50	201.50	273.46
v33	157.64	157.64	157.64	227.91
v34		-34.99		
v35	3.03	3.03	38.02	-1.50
v36			34.99	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D7: Flux distribution of qualified metabolic network cases for FB07, representing the fermentation protocol in which C/N ratio of 102 and %P of 0% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	A6	A10	A13	A14	A18	A21	A22
v1	100.00	100.00	100.00	100.00	100.00	100.00	100.00
v22	68.49	68.49	68.49	68.88	68.49	68.49	68.83
v27	42.53	42.53	42.53	42.75	42.53	42.53	42.72
v31	17.97	17.97	17.97	18.09	17.97	17.97	18.08
v2	94.72	99.11	99.11	98.87	98.81	98.81	98.41
v3	92.42	96.81	96.81	96.48	96.51	96.51	96.03
v4	98.52	94.13	94.13	93.32	94.43	94.43	93.81
v5	92.14	87.75	87.75	86.83	88.05	88.05	87.33
v6	87.58	83.19	87.58	81.62	83.49	87.58	82.21
v7	-4.09	0.30	0.30	0.45			
v8	-4.39				-0.30	-0.30	-0.50
v9	2.30	2.30	2.30	2.24	2.30	2.30	2.24
v10	1.53	1.53	1.53	1.61	1.53	1.53	1.60
v14	167.97	167.97	167.97	165.92	167.97	167.97	166.09
v16	30.98	30.98	30.98	28.00	30.98	30.98	28.30
v17	30.98	30.98	30.98	27.84	30.98	30.98	28.17
v18	36.25	36.25	36.25	32.97	36.25	36.25	33.31
v19	36.25	36.25	36.25	32.90	36.25	36.25	33.25
v20	36.25	36.25	40.63	32.82	36.25	40.33	33.18
v21	63.19	63.19	63.19	63.63	63.19	63.19	63.57
v25	26.68	26.68	26.68	26.90	26.68	26.68	26.87
v26	21.22	21.22	21.22	21.44	21.22	21.22	21.40
v28	176.12	176.12	176.12	169.18	176.12	176.12	169.86
v29	18.12	18.12	18.12	16.45	18.12	18.12	16.62
v30	667.06	658.28	662.67	620.40	658.88	662.97	625.00
v32	246.24	246.24	246.24	237.21	246.24	246.24	238.12
v33	194.25	194.25	194.25	185.63	194.25	194.25	186.47
v34		4.39			4.09		
v35	2.35	2.35	-2.04	2.92	2.35	-1.74	2.84
v36			-4.39			-4.09	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D8: Flux distribution of qualified metabolic network cases for FB08, representing the fermentation protocol in which C/N ratio of 102 and %P of 100% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	B10	B13	B14
v11	100.00	100.00	100.00
v22	22.16	22.16	19.27
v24	11.77	11.77	8.97
v27	23.20	23.20	21.67
v31	14.94	14.94	13.86
v2	1.25	1.25	2.96
v3			
v4	-4.94	-4.94	1.66
v5	-8.42	-8.42	-0.68
v6	-13.48	-66.13	3.61
v7	1.90	1.90	-0.02
v8	1.74	1.74	1.63
v9	1.25	1.25	3.86
v10	0.84	0.84	-1.01
v12	91.70	91.70	94.81
v13	91.70	91.70	96.60
v14	71.65	71.65	94.98
v15			
v16	13.82	13.82	47.81
v17	13.82	13.82	49.61
v18	14.12	14.12	51.73
v19	105.82	105.82	149.23
v20	105.82	53.17	150.13
v21	20.43	20.43	16.93
v23	10.04	10.04	6.63
v25	17.12	17.12	15.05
v26	15.86	15.86	13.84
v28	70.62	70.62	152.63
v29	52.91	52.91	74.61
v30	172.79	120.14	613.85
v32	103.06	103.06	210.37
v33	123.53	123.53	227.25
v34	-52.65		
v35	3.85	56.50	-4.53
v36		52.65	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D9: Flux distribution of qualified metabolic network cases for FB09, representing the fermentation protocol in which C/N ratio of 102 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	74.41	74.41	74.41	74.36
v11	25.59	25.59	25.59	25.64
v22	11.17	11.17	11.17	18.55
v24	5.22	5.22	5.22	12.08
v27	28.62	28.62	28.62	34.36
v31	12.52	12.52	12.52	15.84
v2	-66.92	73.81	73.81	67.21
v3	-68.46	72.27	72.27	63.21
v4	211.19	70.46	70.46	48.20
v5	206.90	66.17	66.17	40.91
v6	203.62	62.88	203.62	15.15
v7	-140.52	0.20	0.20	4.52
v8	-140.72			
v9	1.55	1.55	1.55	-0.28
v10	1.03	1.03	1.03	3.38
v12	24.66	24.66	24.66	17.41
v13	24.66	24.66	24.66	13.13
v14	151.71	151.71	151.71	79.62
v15				
v16	122.01	122.01	122.01	23.15
v17	122.01	122.01	122.01	18.87
v18	125.34	125.34	125.34	18.18
v19	150.00	150.00	150.00	29.16
v20	150.00	150.00	290.73	27.02
v21	9.03	9.03	9.03	17.69
v23	3.07	3.07	3.07	11.23
v25	18.16	18.16	18.16	24.36
v26	14.64	14.64	14.64	20.39
v28	323.59	323.59	323.59	91.87
v29	75.00	75.00	75.00	14.58
v30	1752.23	1470.77	1611.50	216.58
v32	406.29	406.29	406.29	103.51
v33	398.59	398.59	398.59	106.45
v34		140.72		
v35	1.79	1.79	-138.93	21.84
v36			-140.72	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D10: Flux distribution of qualified metabolic network cases for FB10, representing the fermentation protocol in which C/N ratio of 102 and %P of 50% was successfully controlled.

Flux	C14	C26	C29	C30
v1	75.22	75.22	75.22	75.23
v11	24.78	24.78	24.78	24.77
v22	29.52	29.52	29.52	32.88
v24	23.51	23.51	23.51	26.68
v27	10.83	10.83	10.83	12.42
v31	18.16	18.16	18.16	19.59
v2	19.36	75.00	75.00	72.55
v3	18.78	74.41	74.41	71.08
v4	129.36	73.72	73.72	65.47
v5	127.73	72.10	72.10	62.80
v6	119.78	64.15	119.78	46.25
v7	-55.56	0.08	0.08	1.70
v8	-55.63			
v9	0.59	0.59	0.59	-0.13
v10	0.39	0.39	0.39	1.25
v12	2.89	2.89	2.89	-0.54
v13	2.89	2.89	2.89	-2.14
v14	138.39	138.39	138.39	110.87
v15				
v16	55.02	55.02	55.02	15.72
v17	55.02	55.02	55.02	14.11
v18	49.58	49.58	49.58	6.86
v19	52.47	52.47	52.47	3.91
v20	52.47	52.47	108.10	3.11
v21	28.71	28.71	28.71	32.60
v23	22.69	22.69	22.69	26.39
v25	13.59	13.59	13.59	15.76
v26	16.72	16.72	16.72	18.95
v28	186.59	186.59	186.59	95.09
v29	26.24	26.24	26.24	1.96
v30	936.29	825.03	880.65	332.86
v32	239.02	239.02	239.02	119.84
v33	212.83	212.83	212.83	97.05
v34		55.63		
v35	7.39	7.39	-48.25	15.10
v36			-55.63	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D11: Flux distribution of qualified metabolic network cases for FB11, representing the fermentation protocol in which C/N ratio of 102 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	68.29	68.29	68.29	68.12
v11	31.71	31.71	31.71	31.88
v22	16.89	16.89	16.89	19.16
v24	8.57	8.57	8.57	10.63
v27	59.30	59.30	59.30	61.71
v31	21.02	21.02	21.02	22.18
v2	24.05	67.04	67.04	64.98
v3	20.85	63.84	63.84	61.03
v4	103.09	60.11	60.11	53.44
v5	94.20	51.21	51.21	43.56
v6	89.86	46.87	89.86	32.71
v7	-42.57	0.42	0.42	1.67
v8	-42.99			
v9	3.20	3.20	3.20	2.72
v10	2.14	2.14	2.14	2.84
v12	32.01	32.01	32.01	30.11
v13	32.01	32.01	32.01	28.87
v14	125.94	125.94	125.94	104.53
v15				
v16	79.16	79.16	79.16	49.60
v17	79.16	79.16	79.16	48.36
v18	88.51	88.51	88.51	56.71
v19	120.53	120.53	120.53	84.97
v20	120.53	120.53	163.51	84.35
v21	12.46	12.46	12.46	15.04
v23	4.13	4.13	4.13	6.51
v25	35.17	35.17	35.17	37.38
v26	26.23	26.23	26.23	28.18
v28	228.29	228.29	228.29	159.14
v29	60.26	60.26	60.26	42.48
v30	945.20	859.23	902.21	482.55
v32	311.04	311.04	311.04	221.41
v33	288.56	288.56	288.56	201.62
v34		42.99		
v35	1.26	1.26	-41.73	7.03
v36			-42.99	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D12: Flux distribution of qualified metabolic network cases for FB12, representing the fermentation protocol in which C/N ratio of 102 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	75.82	75.82	75.82	75.90
v11	24.18	24.18	24.18	24.10
v22	5.00	5.00	5.00	12.85
v24	4.53	4.53	4.53	12.35
v27	34.04	34.04	34.04	41.14
v31	8.32	8.32	8.32	11.72
v2	-84.43	75.11	75.11	67.67
v3	-86.27	73.27	73.27	62.99
v4	230.66	71.13	71.13	45.67
v5	225.56	66.02	66.02	37.04
v6	224.94	65.40	224.94	10.90
v7	-159.30	0.24	0.24	5.20
v8	-159.53			
v9	1.83	1.83	1.83	-0.23
v10	1.23	1.23	1.23	3.94
v12	24.74	24.74	24.74	16.44
v13	24.74	24.74	24.74	11.52
v14	153.78	153.78	153.78	71.31
v15				
v16	136.71	136.71	136.71	24.78
v17	136.71	136.71	136.71	19.87
v18	143.95	143.95	143.95	22.88
v19	168.69	168.69	168.69	31.95
v20	168.69	168.69	328.23	29.50
v21	2.45	2.45	2.45	11.74
v23	1.99	1.99	1.99	11.23
v25	18.32	18.32	18.32	25.43
v26	11.93	11.93	11.93	18.27
v28	353.86	353.86	353.86	91.10
v29	84.35	84.35	84.35	15.98
v30	1931.84	1612.77	1772.30	188.43
v32	446.31	446.31	446.31	103.03
v33	438.20	438.20	438.20	107.08
v34		159.53		
v35	-1.15	-1.15	-160.69	21.54
v36			-159.53	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its directionality of irreversibility and physiological sense, respectively.

Table D13: Flux distribution of qualified metabolic network cases for FB13, representing the fermentation protocol in which C/N ratio of 102 and %P of 50% was successfully controlled. All flux values are given in mol / total mol carbon consumed.

Flux	C14	C26	C29	C30
v1	57.37	57.37	57.37	57.10
v11	42.63	42.63	42.63	42.90
v22	10.00	10.00	10.00	11.26
v24	3.83	3.83	3.83	4.98
v27	70.99	70.99	70.99	72.60
v31	32.23	32.23	32.23	33.09
v2	30.02	55.87	55.87	54.48
v3	26.19	52.04	52.04	50.19
v4	73.42	47.57	47.57	43.41
v5	62.78	36.92	36.92	32.16
v6	54.04	28.19	54.04	19.48
v7	-25.36	0.50	0.50	1.25
v8	-25.85			
v9	3.83	3.83	3.83	3.55
v10	2.56	2.56	2.56	2.98
v12	49.43	49.43	49.43	48.55
v13	49.43	49.43	49.43	47.82
v14	109.57	109.57	109.57	96.58
v15				
v16	80.43	80.43	80.43	62.84
v17	80.43	80.43	80.43	62.10
v18	88.10	88.10	88.10	69.15
v19	137.53	137.53	137.53	116.60
v20	137.53	137.53	163.38	116.23
v21	4.70	4.70	4.70	6.12
v23	-1.49	-1.49	-1.49	-0.15
v25	45.65	45.65	45.65	47.12
v26	37.29	37.29	37.29	38.61
v28	215.39	215.39	215.39	174.01
v29	68.76	68.76	68.76	58.30
v30	785.58	733.87	759.72	507.66
v32	295.42	295.42	295.42	241.80
V33	284.15	284.15	284.15	232.31
V34		25.85		
V35	5.04	5.04	-20.82	8.53
V36			-25.85	

Note: The bold and light highlights indicate the corresponding flux value is inconsistent with its irreversible reaction and physiological sense, respectively.

Table D14: Summary of estimated non-measured rates of all fermentation protocols.

Run	CO_2 evolution rate (m mol / g RB / h)	O_2 uptake rate (m mol / g RB / h)	RQ	Carbon recovery (%)
FB01	0.4842	0.4491	1.0782	93.09
FB02	0.3057	0.2748	1.1124	76.10
FB03	0.5429	0.5408	1.0039	105.86
FB04	0.1795	0.1873	0.9584	82.54
FB05	0.2088	0.2727	0.7657	52.21
FB06	0.3260	0.2551	1.2779	126.42
FB07	0.7192	0.5628	1.2779	113.94
FB08	0.1838	0.2204	0.8339	114.42
FB09	0.3474	0.3573	0.9723	71.83
FB10	0.2469	0.2000	1.2345	82.69
FB11	0.5800	0.5282	1.0981	118.58
FB12	0.3932	0.4086	0.9623	72.12
FB13	0.4985	0.4789	1.0409	127.83

Table D15: Yield values calculated from the reconciled measured rate.

Yield	FB01	FB02	FB03	FB04	FB05	FB06	FB07	FB8	FB09	FB10	FB11	FB12	FB13
$Y_{3HB/G}$	0.2113	0.4015	0.1549	0.1692	0.1218	0.5872	0.6887		0.2497	0.4374	0.2812	0.1691	0.1971
$Y_{3HV/P}$	0.5472	0.9345	0.1494	0.3243	0.3438	0.5413		0.1177	0.4715	1.0784	0.3341	0.5120	0.1165
3HV fraction (%)	27.40	21.51	36.95	53.22	31.05	20.34	0.00	34.71	39.46	44.79	35.72	49.01	30.75
Polymer content (%)	26.87	57.07	17.84	33.27	25.36	46.91	58.08	57.04	44.96	81.57	30.53	36.24	16.81

Note: All values are given in molar basis except for values of polymer content that are given in mass basis.
$Y_{3HB/G}$ is defined as 3HB formation rate / glucose uptake rate
$Y_{3HV/P}$ is defined as 3HV formation rate / propionic acid uptake rate
3HV fraction is defined as 100 * 3HV formation rate / (3HB formation rate +3HV formation rate)
Polymer content is defined as 100 * (3HB formation rate +3HV formation rate) / (RB formation rate + 3HB formation rate +3HV formation rate)

Table D16: Theoretical flux split ratios at all nodes in the metabolic network.

Node	Flux split ratio	Molar ratio of glucose uptake rate to propionic acid uptake rate								
		9:1	7:3	7:3	7:3	5:5	5:5	5:5	5:5	5:5
	HV fraction	10.71	12.50	25.00	37.50	15.00	30.00	45.00	60.00	75.00
G6P	v2/v1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v27/v1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GN6P	v3/v2	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v9/v2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GA3P	v4/v3	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v7/v3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	v10/v3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	v27/v3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F6P	v10/v7	50.00	#DIV/0!	#DIV/0!	#DIV/0!	33.33	33.33	33.33	33.33	33.33
	v27/v7	0.47	#DIV/0!	#DIV/0!	#DIV/0!	0.47	0.47	0.47	0.47	0.47
G3P	v5/v4	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v27/v4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table D16 (cont.): Theoretical flux split ratios at all nodes in the metabolic network.

Node	Flux split ratio	Molar ratio of glucose uptake rate to propionic acid uptake rate								
		9:1	7:3	7:3	7:3	5:5	5:5	5:5	5:5	5:5
PEP	v6/v5	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v27/v5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	v35/v5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PYR	v14/(v3+v6+v13)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v27/(v3+v6+v13)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AcCoA	v16/v14	1.85	6.25	6.67	7.14	11.90	12.82	13.89	15.15	16.67
	2v21/v14	92.59	87.50	80.00	71.43	80.95	71.80	61.11	48.49	33.34
	v23/v14	5.55	6.25	13.33	21.43	7.14	15.38	25.00	36.36	50.00
ProCoA	v12/v11	0.01	66.67	33.33	0.00	80.00	60.00	40.00	20.00	0.00
	v23/v11	99.99	33.33	66.67	100.00	20.00	40.00	60.00	80.00	100.00

Table D16 (cont.): Theoretical flux split ratios at all nodes in the metabolic network.

Node	Flux split ratio	Molar ratio of glucose uptake rate to propionic acid uptake rate								
		9:1	7:3	7:3	7:3	5:5	5:5	5:5	5:5	5:5
AKG	v18/(v17+v27)	100.02	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	v25/(v17+v27)	-0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OAA	v12/v20	0.04	66.67	50.00	0.00	70.59	64.28	54.54	37.50	0.00
	v16/v20	99.90	33.33	50.00	100.00	29.41	35.71	45.45	62.50	99.99
	v27/v20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLUT	v26/v25	100.00	#DIV/0!	#DIV/0!	#DIV/0!	100.00	100.00	100.00	100.00	100.00
	v27/v25	1.67	#DIV/0!	#DIV/0!	#DIV/0!	1.67	1.67	1.67	1.67	1.67

Note: #DIV/0! indicates that the value cannot be determined since the incoming flux is zero.

APPENDIX E:

Source code m-files

E1. MFA.m
E2. analyze_G.m

In this appendix, the source code m-files that are used in this research are shown. The first one, MFA.m, is used for flux calculation according to section 5.1. While analyze_G.m is used for determining the characteristics of a metabolic network. In deed, it follows the concept of metabolic pathway analysis according to section 4.5. These two source code m-files are running in purpose with MATLAB 6.1.

E1. MFA.m

```
%-------------------------------------------------------------------------
% METABOLIC FLUX ANALYSIS OF PHA BIOSYNTHETIC
PATHWAYS IN R.EUTROPHA NCIMB 11599
%-------------------------------------------------------------------------

clear all

% 1. Stoichiometric matrices setting up

% Group 1  : Substrate uptake          v1,  v11, v31
% Group 2  : Product formation         v22, v24, v27
% Group 3  : cyclic ED pathway         v2 - v8
% Group 4  : simplified PP pathway     v9 - v10
% Group 5  : gluconeogenesis           v2 - v8 (except v3)
% Group 6  : methylcitrate pathway     v12 - v13
% Group 7  : acetyl-CoA formation      v14 - v15
% Group 8  : TCA cycle                 v16 - v20
% Group 9  : PHBV formation            v21, v23
% Group 10 : GS-GOGAT pathway          v25 - v26
% Group 11 : oxidative phosphorylation  v28 - v29
% Group 12 : maintenance               v30
% Group 13 : respiratory system        v32 - v33
% Group 14 : transhydrogenation        v34
% Group 15 : anaplerotic pathway       v35 - v36

% 1.1 Declaration of all elements of stoichiometric matrices

G6P   = zeros(36,1); GN6P   = zeros(36,1); GA3P   = zeros(36,1);
F6P   = zeros(36,1);
G3P   = zeros(36,1); PEP    = zeros(36,1); PYR    = zeros(36,1);
R5P   = zeros(36,1);
E4P   = zeros(36,1); ProCoA = zeros(36,1); MetCit = zeros(36,1);
AcCoA = zeros(36,1);
IsoCit = zeros(36,1); AKG    = zeros(36,1); SUC    = zeros(36,1);
MAL   = zeros(36,1);
OAA   = zeros(36,1); AcAcCoA = zeros(36,1); KetVaCoA =
zeros(36,1); GLUT  = zeros(36,1);
GLUM  = zeros(36,1); NH3    = zeros(36,1); CO2    =
zeros(36,1); O2    = zeros(36,1);
ATP   = zeros(36,1); NADH   = zeros(36,1); NADPH   =
zeros(36,1); FADH2 = zeros(36,1);
CoA   = zeros(36,1);

% 1.2 Stoichiometric coefficients

G6P(1)  = 1; G6P(2)  = -1; G6P(8)  =  1;   G6P(27)  = -0.021;
GN6P(2) = 1; GN6P(3) = -1; GN6P(9) = -1;
GA3P(3) = 1; GA3P(4) = -1; GA3P(7) = -2;   GA3P(10) = -1;
GA3P(27) = -0.013;
F6P(7)  = 1; F6P(8)  = -1; F6P(27) = -0.007;
G3P(4)  = 1; G3P(5)  = -1; G3P(27) = -0.15;
PEP(5)  = 1; PEP(6)  = -1; PEP(27) = -0.052; PEP(35) = -1;
PYR(3)  = 1; PYR(6)  =  1; PYR(13) =  1;    PYR(14) = -1;
PYR(27) = -0.283; PYR(36) = 1;
R5P(9)  = 1; R5P(10) =  1; R5P(27) = -0.09;
E4P(10) = 1; E4P(27) = -0.036;
ProCoA(11) = 1; ProCoA(12) = -1; ProCoA(15)= -1; ProCoA(23)=
-1;
MetCit(12) = 1; MetCit(13) = -1;
AcCoA(14)  = 1; AcCoA(15)  =  1; AcCoA(16) = -1; AcCoA(21) =
-2; AcCoA(23) = -1; AcCoA(27) = -0.374;
```

```
IsoCit(16) = 1; IsoCit(17) = -1;
AKG(17) =  1; AKG(18) = -1; AKG(25) = -1; AKG(27) = 0.751;
SUC(13) =  1; SUC(18) =  1; SUC(19) = -1;
MAL(19) =  1; MAL(20) = -1; MAL(36) = -1;
OAA(12) = -1; OAA(16) = -1; OAA(20) =  1; OAA(27) = -0.179;
OAA(35) = 1;
AcAcCoA(21)  = 1; AcAcCoA(22)  = -1;
KetVaCoA(23) = 1; KetVaCoA(24) = -1;
GLUT(25)=  2; GLUT(26) = -1; GLUT(27) = -0.832;
GLUM(25)= -1; GLUM(26) =  1; GLUM(27) = -0.025;
NH3(26) = -1; NH3(31)  =  1;
CO2(9)  =  1; CO2(14)  =  1; CO2(15) = 1; CO2(17) = 1; CO2(18) =
1; CO2(27) = 0.261; CO2(32) = -1; CO2(35) = -1; CO2(36) = 1;
O2(28)  = -1; O2(29)   = -1; O2(33)  = 1;
ATP(1)  = -1; ATP(4)   =  1; ATP(6)  = 1; ATP(11) = -1; ATP(18) =
1; ATP(26) = -1; ATP(27) = -4.11; ATP(28) = 4; ATP(29) = 2;
ATP(30) = -1;
NADH(4) =  1; NADH(14) =  1; NADH(18)= 1; NADH(20)=  1;
NADH(27)= 0.312; NADH(28) = -2; NADH(34) = 1;
NADPH(2)  = 1; NADPH(9) = 2; NADPH(17) = 1; NADPH(22) = -
1; NADPH(24) = -1; NADPH(25) = -1; NADPH(27) = -0.826;
NADPH(34) = -1; NADPH(36) = 1;
FADH2(19) = 1; FADH2(29) = -2;
CoA(11) = -1; CoA(12) = 1; CoA(14) = -1; CoA(16) = 1; CoA(21)
= 1; CoA(22) = 1; CoA(23) = 1; CoA(24) = 1;

% 1.3 Stoichiometric matrix

G_all = [G6P GN6P GA3P F6P G3P PEP PYR R5P E4P ProCoA
MetCit AcCoA IsoCit AKG SUC MAL OAA AcAcCoA KetVaCoA
GLUT GLUM NH3 CO2 O2 ATP NADH NADPH FADH2 CoA];

% 2. Model classification

Choice1 = input('What is the substrate of interest? (1:glucose
2:propionic acid 3:both) : ');
fprintf('\n');

% 2.1 Metabolic network case A

if Choice1 == 1
  GmA  = [G_all(1,    1:9) G_all(1,    12:18) G_all(1,    20:29);
          G_all(22,   1:9) G_all(22,   12:18) G_all(22,   20:29);
          G_all(27,   1:9) G_all(27,   12:18) G_all(27,   20:29);
          G_all(31,   1:9) G_all(31,   12:18) G_all(31,   20:29)];

  Gc_caseA32 = [G_all(2:6,  1:9) G_all(2:6,  12:18) G_all(2:6,
20:29);
                G_all(9:10, 1:9) G_all(9:10, 12:18) G_all(9:10, 20:29);
                G_all(14,   1:9) G_all(14,   12:18) G_all(14,   20:29);
                G_all(16:21,1:9) G_all(16:21,12:18) G_all(16:21,20:29);
                G_all(25:26,1:9) G_all(25:26,12:18) G_all(25:26,20:29);
                G_all(28:30,1:9) G_all(28:30,12:18) G_all(28:30,20:29);
                G_all(32:33,1:9) G_all(32:33,12:18) G_all(32:33,20:29)];
  Gc_caseA31 = [Gc_caseA32;
                G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA30 = [Gc_caseA32;
                G_all(35,   1:9) G_all(35,   12:18) G_all(35,   20:29)];
Gc_caseA29 = [Gc_caseA32;
                G_all(35:36,1:9) G_all(35:36,12:18) G_all(35:36,20:29)];
  Gc_caseA28 = [Gc_caseA32;
                G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29)];
Gc_caseA27 = [Gc_caseA32;
                G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29);
```

```
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA26 = [Gc_caseA32;
        G_all(34:35,1:9) G_all(34:35,12:18) G_all(34:35,20:29)];
  Gc_caseA25 = [Gc_caseA32;
        G_all(34:36,1:9) G_all(34:36,12:18) G_all(34:36,20:29)];

  Gc_caseA24 = [G_all(2:6,  1:9) G_all(2:6,  12:18) G_all(2:6,
20:29);
        G_all(8:10, 1:9) G_all(8:10, 12:18) G_all(8:10, 20:29);
        G_all(14,   1:9) G_all(14,   12:18) G_all(14,   20:29);
        G_all(16:21,1:9) G_all(16:21,12:18) G_all(16:21,20:29);
        G_all(25:26,1:9) G_all(25:26,12:18) G_all(25:26,20:29);
        G_all(28:30,1:9) G_all(28:30,12:18) G_all(28:30,20:29);
        G_all(32:33,1:9) G_all(32:33,12:18) G_all(32:33,20:29)];
  Gc_caseA23 = [Gc_caseA24;
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA22 = [Gc_caseA24;
        G_all(35,   1:9) G_all(35,   12:18) G_all(35,   20:29)];
  Gc_caseA21 = [Gc_caseA24;
        G_all(35:36,1:9) G_all(35:36,12:18) G_all(35:36,20:29)];
  Gc_caseA20 = [Gc_caseA24;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29)];
  Gc_caseA19 = [Gc_caseA24;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29);
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA18 = [Gc_caseA24;
        G_all(34:35,1:9) G_all(34:35,12:18) G_all(34:35,20:29)];
  Gc_caseA17 = [Gc_caseA24;
        G_all(34:36,1:9) G_all(34:36,12:18) G_all(34:36,20:29)];

  Gc_caseA16 = [G_all(2:7,  1:9) G_all(2:7,  12:18) G_all(2:7,
20:29);
        G_all(9:10, 1:9) G_all(9:10, 12:18) G_all(9:10, 20:29);
        G_all(14,   1:9) G_all(14,   12:18) G_all(14,   20:29);
        G_all(16:21,1:9) G_all(16:21,12:18) G_all(16:21,20:29);
        G_all(25:26,1:9) G_all(25:26,12:18) G_all(25:26,20:29);
        G_all(28:30,1:9) G_all(28:30,12:18) G_all(28:30,20:29);
        G_all(32:33,1:9) G_all(32:33,12:18) G_all(32:33,20:29)];
  Gc_caseA15 = [Gc_caseA16;
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA14 = [Gc_caseA16;
        G_all(35,   1:9) G_all(35,   12:18) G_all(35,   20:29)];
  Gc_caseA13 = [Gc_caseA16;
        G_all(35:36,1:9) G_all(35:36,12:18) G_all(35:36,20:29)];
  Gc_caseA12 = [Gc_caseA16;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29)];
  Gc_caseA11 = [Gc_caseA16;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29);
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA10 = [Gc_caseA16;
        G_all(34:35,1:9) G_all(34:35,12:18) G_all(34:35,20:29)];
  Gc_caseA9  = [Gc_caseA16;
        G_all(34:36,1:9) G_all(34:36,12:18) G_all(34:36,20:29)];

  Gc_caseA8  = [G_all(2:10, 1:9) G_all(2:10, 12:18) G_all(2:10,
20:29);
        G_all(14,   1:9) G_all(14,   12:18) G_all(14,   20:29);
        G_all(16:21,1:9) G_all(16:21,12:18) G_all(16:21,20:29);
        G_all(25:26,1:9) G_all(25:26,12:18) G_all(25:26,20:29);
        G_all(28:30,1:9) G_all(28:30,12:18) G_all(28:30,20:29);
        G_all(32:33,1:9) G_all(32:33,12:18) G_all(32:33,20:29)];
  Gc_caseA7  = [Gc_caseA8;
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA6  = [Gc_caseA8;
```

```
        G_all(35,   1:9) G_all(35,   12:18) G_all(35,   20:29)];
  Gc_caseA5  = [Gc_caseA8;
        G_all(35:36,1:9) G_all(35:36,12:18) G_all(35:36,20:29)];
  Gc_caseA4  = [Gc_caseA8;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29)];
  Gc_caseA3  = [Gc_caseA8;
        G_all(34,   1:9) G_all(34,   12:18) G_all(34,   20:29);
        G_all(36,   1:9) G_all(36,   12:18) G_all(36,   20:29)];
  Gc_caseA2  = [Gc_caseA8;
        G_all(34:35,1:9) G_all(34:35,12:18) G_all(34:35,20:29)];
  Gc_caseA1  = [Gc_caseA8;
        G_all(34:36,1:9) G_all(34:36,12:18) G_all(34:36,20:29)];

% 2.2 Metabolic network case B

elseif Choice1 == 2
  GmB = [G_all(11,:);
      G_all(22,:);
      G_all(24,:);
      G_all(27,:);
      G_all(31,:)];

  Gc_caseB16 = [G_all(2,    :);
        G_all(4:10, :);
        G_all(12:14,:);
        G_all(16:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
  Gc_caseB15 = [Gc_caseB16;
        G_all(36,   :)];
Gc_caseB14 = [Gc_caseB16;
        G_all(35,   :)];
Gc_caseB13 = [Gc_caseB16;
        G_all(35:36,:)];
Gc_caseB12 = [Gc_caseB16;
        G_all(34,   :)];
Gc_caseB11 = [Gc_caseB16;
        G_all(34,   :);
        G_all(36,   :)];
Gc_caseB10 = [Gc_caseB16;
        G_all(34:35,:)];
Gc_caseB9  = [Gc_caseB16;
        G_all(34:36,:)];

Gc_caseB8  = [G_all(2,    :);
        G_all(4:10, :);
        G_all(12:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseB7  = [Gc_caseB8;
        G_all(36,   :)];
Gc_caseB6  = [Gc_caseB8;
        G_all(35,   :)];
Gc_caseB5  = [Gc_caseB8;
        G_all(35:36,:)];
Gc_caseB4  = [Gc_caseB8;
        G_all(34,   :)];
Gc_caseB3  = [Gc_caseB8;
        G_all(34,   :);
        G_all(36,   :)];
```

```
   Gc_caseB2  = [Gc_caseB8;
                G_all(34:35,:)];
   Gc_caseB1  = [Gc_caseB8;
                G_all(34:36,:)];

% 2.3 Metabolic network case C

elseif Choice1 == 3
   GmC = [G_all(1, :);
          G_all(11,:);
          G_all(22,:);
          G_all(24,:);
          G_all(27,:);
          G_all(31,:)];

   Gc_caseC64 = [G_all(2:6,  :);
                G_all(9:10, :);
                G_all(12:14,:);
                G_all(16:21,:);
                G_all(23,   :);
                G_all(25:26,:);
                G_all(28:30,:);
                G_all(32:33,:)];
   Gc_caseC63 = [Gc_caseC64;
                G_all(36,   :)];
   Gc_caseC62 = [Gc_caseC64;
                G_all(35,   :)];
   Gc_caseC61 = [Gc_caseC64;
                G_all(35:36,:)];
   Gc_caseC60 = [Gc_caseC64;
                G_all(34,   :)];
   Gc_caseC59 = [Gc_caseC64;
                G_all(34,   :);
                G_all(36,   :)];
   Gc_caseC58 = [Gc_caseC64;
                G_all(34:35,:)];
   Gc_caseC57 = [Gc_caseC64;
                G_all(34:36,:)];

   Gc_caseC56 = [G_all(2:6,  :);
                G_all(9:10, :);
                G_all(12:21,:);
                G_all(23,   :);
                G_all(25:26,:);
                G_all(28:30,:);
                G_all(32:33,:)];
   Gc_caseC55 = [Gc_caseC56;
                G_all(36,   :)];
   Gc_caseC54 = [Gc_caseC56;
                G_all(35,   :)];
   Gc_caseC53 = [Gc_caseC56;
                G_all(35:36,:)];
   Gc_caseC52 = [Gc_caseC56;
                G_all(34,   :)];
   Gc_caseC51 = [Gc_caseC56;
                G_all(34,   :);
                G_all(36,   :)];
   Gc_caseC50 = [Gc_caseC56;
                G_all(34:35,:)];
   Gc_caseC49 = [Gc_caseC56;
                G_all(34:36,:)];

   Gc_caseC48 = [G_all(2:6,  :);
                G_all(8:10, :);
```

```
        G_all(12:14,:);
        G_all(16:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC47 = [Gc_caseC48;
        G_all(36,   :)];
Gc_caseC46 = [Gc_caseC48;
        G_all(35,   :)];
Gc_caseC45 = [Gc_caseC48;
        G_all(35:36,:)];
Gc_caseC44 = [Gc_caseC48;
        G_all(34,   :)];
Gc_caseC43 = [Gc_caseC48;
        G_all(34,   :);
        G_all(36,   :)];
Gc_caseC42 = [Gc_caseC48;
        G_all(34:35,:)];
Gc_caseC41 = [Gc_caseC48;
        G_all(34:36,:)];

Gc_caseC40 = [G_all(2:6,  :);
        G_all(8:10, :);
        G_all(12:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC39 = [Gc_caseC40;
        G_all(36,   :)];
Gc_caseC38 = [Gc_caseC40;
        G_all(35,   :)];
Gc_caseC37 = [Gc_caseC40;
        G_all(35:36,:)];
Gc_caseC36 = [Gc_caseC40;
        G_all(34,   :)];
Gc_caseC35 = [Gc_caseC40;
        G_all(34,   :);
        G_all(36,   :)];
Gc_caseC34 = [Gc_caseC40;
        G_all(34:35,:)];
Gc_caseC33 = [Gc_caseC40;
        G_all(34:36,:)];

Gc_caseC32 = [G_all(2:7,  :);
        G_all(9:10, :);
        G_all(12:14,:);
        G_all(16:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC31 = [Gc_caseC32;
        G_all(36,   :)];
Gc_caseC30 = [Gc_caseC32;
        G_all(35,   :)];
Gc_caseC29 = [Gc_caseC32;
        G_all(35:36,:)];
Gc_caseC28 = [Gc_caseC32;
        G_all(34,   :)];
Gc_caseC27 = [Gc_caseC32;
        G_all(34,   :);
        G_all(36,   :)];
```

```
Gc_caseC26 = [Gc_caseC32;
        G_all(34:35,:)];
Gc_caseC25 = [Gc_caseC32;
        G_all(34:36,:)];

Gc_caseC24 = [G_all(2:7,  :);
        G_all(9:10, :);
        G_all(12:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC23 = [Gc_caseC24;
        G_all(36,   :)];
Gc_caseC22 = [Gc_caseC24;
        G_all(35,   :)];
Gc_caseC21 = [Gc_caseC24;
        G_all(35:36,:)];
Gc_caseC20 = [Gc_caseC24;
        G_all(34,   :)];
Gc_caseC19 = [Gc_caseC24;
        G_all(34,   :);
        G_all(36,   :)];
Gc_caseC18 = [Gc_caseC24;
        G_all(34:35,:)];
Gc_caseC17 = [Gc_caseC24;
        G_all(34:36,:)];

Gc_caseC16 = [G_all(2:10, :);
        G_all(12:14,:);
        G_all(16:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC15 = [Gc_caseC16;
        G_all(36,   :)];
Gc_caseC14 = [Gc_caseC16;
        G_all(35,   :)];
Gc_caseC13 = [Gc_caseC16;
        G_all(35:36,:)];
Gc_caseC12 = [Gc_caseC16;
        G_all(34,   :)];
Gc_caseC11 = [Gc_caseC16;
        G_all(34,   :);
        G_all(36,   :)];
Gc_caseC10 = [Gc_caseC16;
        G_all(34:35,:)];
Gc_caseC9  = [Gc_caseC16;
        G_all(34:36,:)];

Gc_caseC8  = [G_all(2:10,  :);
        G_all(12:21,:);
        G_all(23,   :);
        G_all(25:26,:);
        G_all(28:30,:);
        G_all(32:33,:)];
Gc_caseC7  = [Gc_caseC8;
        G_all(36,   :)];
Gc_caseC6  = [Gc_caseC8;
        G_all(35,   :)];
Gc_caseC5  = [Gc_caseC8;
        G_all(35:36,:)];
Gc_caseC4  = [Gc_caseC8;
```

```
            G_all(34,   :)];
  Gc_caseC3  = [Gc_caseC8;
            G_all(34,   :);
            G_all(36,   :)];
  Gc_caseC2  = [Gc_caseC8;
            G_all(34:35,:)];
  Gc_caseC1  = [Gc_caseC8;
            G_all(34:36,:)];

else
  disp('You did not enter 1,2 or 3 : start over');
end

% 3.Specification of all measured rates

% 3.1 Molecular weight information

MW_G  = 180;
MW_P  =  74;
MW_N  = 132;
MW_HB =  86;
MW_HV = 100;
MW_RB = 100;

% 3.2 Unnormalized measured rates
            %Y6        Y7        Y2        Y3        Y1
Y8
            %v1        v11       v22       v24       v27
v31

vm1_unnorm  = [0.0484/MW_G; 0.0031/MW_P; 0.0034/MW_HB;
0.0006/MW_HV; 0.0188/MW_RB; 0.0105/MW_N];
vm2_unnorm  = [0.0447/MW_G; 0.0024/MW_P; 0.0068/MW_HB;
0.0011/MW_HV; 0.0078/MW_RB; 0.0048/MW_N];
vm3_unnorm  = [0.0337/MW_G; 0.0082/MW_P; 0.0014/MW_HB;
0.0005/MW_HV; 0.0195/MW_RB; 0.0060/MW_N];
vm4_unnorm  = [0.0170/MW_G; 0.0040/MW_P; 0.0006/MW_HB;
0.0009/MW_HV; 0.0061/MW_RB; 0.0016/MW_N];
vm5_unnorm  = [0.0519/MW_G; 0.0035/MW_P; 0.0023/MW_HB;
0.0008/MW_HV; 0.0086/MW_RB; 0.0628/MW_N];
vm6_unnorm  = [0.0228/MW_G; 0.0026/MW_P; 0.0064/MW_HB;
0.0019/MW_HV; 0.0103/MW_RB; 0.0042/MW_N];
vm7_unnorm  = [0.0547/MW_G;          0.0179/MW_HB;
0.0144/MW_RB; 0.0059/MW_N];
vm8_unnorm  = [          0.0132/MW_P; 0.0034/MW_HB;
0.0021/MW_HV; 0.0044/MW_RB; 0.0033/MW_N];
vm9_unnorm  = [0.0488/MW_G; 0.0069/MW_P; 0.0035/MW_HB;
0.0019/MW_HV; 0.0114/MW_RB; 0.0050/MW_N];
vm10_unnorm = [0.0288/MW_G; 0.0039/MW_P; 0.0054/MW_HB;
0.0050/MW_HV; 0.0021/MW_RB; 0.0055/MW_N];
vm11_unnorm = [0.0330/MW_G; 0.0063/MW_P; 0.0039/MW_HB;
0.0023/MW_HV; 0.0176/MW_RB; 0.0056/MW_N];
vm12_unnorm = [0.0572/MW_G; 0.0075/MW_P; 0.0018/MW_HB;
0.0019/MW_HV; 0.0160/MW_RB; 0.0026/MW_N];
vm13_unnorm = [0.0216/MW_G; 0.0066/MW_P; 0.0018/MW_HB;
0.0008/MW_HV; 0.0162/MW_RB; 0.0075/MW_N];

% 3.3 Normalized measured rates

vm1 = vm1_unnorm./vm1_unnorm(1);
vm2 = vm2_unnorm./vm2_unnorm(1);
vm3 = vm3_unnorm./vm3_unnorm(1);
vm4 = vm4_unnorm./vm4_unnorm(1);
vm5 = vm5_unnorm./vm5_unnorm(1);
```

```
vm6 = vm6_unnorm./vm6_unnorm(1);
vm7 = vm7_unnorm./vm7_unnorm(1);
vm8 = vm8_unnorm./vm8_unnorm(1);
vm9 = vm9_unnorm./vm9_unnorm(1);
vm10 = vm10_unnorm./vm10_unnorm(1);
vm11 = vm11_unnorm./vm11_unnorm(1);
vm12 = vm12_unnorm./vm12_unnorm(1);
vm13 = vm13_unnorm./vm13_unnorm(1);

vm1 = [70; 30; 60.6; 20.2; 0; 0]
% 4. Stoichiometric matrix analysis

% 4.1 Full rank test and condition number

if Choice1 == 1
   analyze_G(1,Gc_caseA1,GmA);
   analyze_G(2,Gc_caseA2,GmA);
   analyze_G(3,Gc_caseA3,GmA);
   analyze_G(4,Gc_caseA4,GmA);
   analyze_G(5,Gc_caseA5,GmA);
   analyze_G(6,Gc_caseA6,GmA);
   analyze_G(7,Gc_caseA7,GmA);
   analyze_G(8,Gc_caseA8,GmA);
   analyze_G(9,Gc_caseA9,GmA);
   analyze_G(10,Gc_caseA10,GmA);
   analyze_G(11,Gc_caseA11,GmA);
   analyze_G(12,Gc_caseA12,GmA);
   analyze_G(13,Gc_caseA13,GmA);
   analyze_G(14,Gc_caseA14,GmA);
   analyze_G(15,Gc_caseA15,GmA);
   analyze_G(16,Gc_caseA16,GmA);
   analyze_G(17,Gc_caseA17,GmA);
   analyze_G(18,Gc_caseA18,GmA);
   analyze_G(19,Gc_caseA19,GmA);
   analyze_G(20,Gc_caseA20,GmA);
   analyze_G(21,Gc_caseA21,GmA);
   analyze_G(22,Gc_caseA22,GmA);
   analyze_G(23,Gc_caseA23,GmA);
   analyze_G(24,Gc_caseA24,GmA);
   analyze_G(25,Gc_caseA25,GmA);
   analyze_G(26,Gc_caseA26,GmA);
   analyze_G(27,Gc_caseA27,GmA);
   analyze_G(28,Gc_caseA28,GmA);
   analyze_G(29,Gc_caseA29,GmA);
   analyze_G(30,Gc_caseA30,GmA);
   analyze_G(31,Gc_caseA31,GmA);
   analyze_G(32,Gc_caseA32,GmA);

elseif Choice1 == 2
   analyze_G(1,Gc_caseB1,GmB);
   analyze_G(2,Gc_caseB2,GmB);
   analyze_G(3,Gc_caseB3,GmB);
   analyze_G(4,Gc_caseB4,GmB);
   analyze_G(5,Gc_caseB5,GmB);
   analyze_G(6,Gc_caseB6,GmB);
   analyze_G(7,Gc_caseB7,GmB);
   analyze_G(8,Gc_caseB8,GmB);
   analyze_G(9,Gc_caseB9,GmB);
   analyze_G(10,Gc_caseB10,GmB);
   analyze_G(11,Gc_caseB11,GmB);
   analyze_G(12,Gc_caseB12,GmB);
   analyze_G(13,Gc_caseB13,GmB);
   analyze_G(14,Gc_caseB14,GmB);
   analyze_G(15,Gc_caseB15,GmB);
```

```
   analyze_G(16,Gc_caseB16,GmB);

elseif Choice1 == 3
   analyze_G(1,Gc_caseC1,GmC);
   analyze_G(2,Gc_caseC2,GmC);
   analyze_G(3,Gc_caseC3,GmC);
   analyze_G(4,Gc_caseC4,GmC);
   analyze_G(5,Gc_caseC5,GmC);
   analyze_G(6,Gc_caseC6,GmC);
   analyze_G(7,Gc_caseC7,GmC);
   analyze_G(8,Gc_caseC8,GmC);
   analyze_G(9,Gc_caseC9,GmC);
   analyze_G(10,Gc_caseC10,GmC);
   analyze_G(11,Gc_caseC11,GmC);
   analyze_G(12,Gc_caseC12,GmC);
   analyze_G(13,Gc_caseC13,GmC);
   analyze_G(14,Gc_caseC14,GmC);
   analyze_G(15,Gc_caseC15,GmC);
   analyze_G(16,Gc_caseC16,GmC);
   analyze_G(17,Gc_caseC17,GmC);
   analyze_G(18,Gc_caseC18,GmC);
   analyze_G(19,Gc_caseC19,GmC);
   analyze_G(20,Gc_caseC20,GmC);
   analyze_G(21,Gc_caseC21,GmC);
   analyze_G(22,Gc_caseC22,GmC);
   analyze_G(23,Gc_caseC23,GmC);
   analyze_G(24,Gc_caseC24,GmC);
   analyze_G(25,Gc_caseC25,GmC);
   analyze_G(26,Gc_caseC26,GmC);
   analyze_G(27,Gc_caseC27,GmC);
   analyze_G(28,Gc_caseC28,GmC);
   analyze_G(29,Gc_caseC29,GmC);
   analyze_G(30,Gc_caseC30,GmC);
   analyze_G(31,Gc_caseC31,GmC);
   analyze_G(32,Gc_caseC32,GmC);
   analyze_G(33,Gc_caseC33,GmC);
   analyze_G(34,Gc_caseC34,GmC);
   analyze_G(35,Gc_caseC35,GmC);
   analyze_G(36,Gc_caseC36,GmC);
   analyze_G(37,Gc_caseC37,GmC);
   analyze_G(38,Gc_caseC38,GmC);
   analyze_G(39,Gc_caseC39,GmC);
   analyze_G(40,Gc_caseC40,GmC);
   analyze_G(41,Gc_caseC41,GmC);
   analyze_G(42,Gc_caseC42,GmC);
   analyze_G(43,Gc_caseC43,GmC);
   analyze_G(44,Gc_caseC44,GmC);
   analyze_G(45,Gc_caseC45,GmC);
   analyze_G(46,Gc_caseC46,GmC);
   analyze_G(47,Gc_caseC47,GmC);
   analyze_G(48,Gc_caseC48,GmC);
   analyze_G(49,Gc_caseC49,GmC);
   analyze_G(50,Gc_caseC50,GmC);
   analyze_G(51,Gc_caseC51,GmC);
   analyze_G(52,Gc_caseC52,GmC);
   analyze_G(53,Gc_caseC53,GmC);
   analyze_G(54,Gc_caseC54,GmC);
   analyze_G(55,Gc_caseC55,GmC);
   analyze_G(56,Gc_caseC56,GmC);
   analyze_G(57,Gc_caseC57,GmC);
   analyze_G(58,Gc_caseC58,GmC);
   analyze_G(59,Gc_caseC59,GmC);
   analyze_G(60,Gc_caseC60,GmC);
   analyze_G(61,Gc_caseC61,GmC);
```

```
   analyze_G(62,Gc_caseC62,GmC);
   analyze_G(63,Gc_caseC63,GmC);
   analyze_G(64,Gc_caseC64,GmC);

else
   disp('You did not enter 1,2 or 3 : start over');
end

% 5.Flux calculation

if Choice1 == 1

   T_caseA6  = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA6 ,1)); GmA' Gc_caseA6' ;];
   T_caseA10 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA10,1)); GmA' Gc_caseA10';];
   T_caseA13 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA13,1)); GmA' Gc_caseA13';];
   T_caseA14 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA14,1)); GmA' Gc_caseA14';];
   T_caseA18 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA18,1)); GmA' Gc_caseA18';];
   T_caseA21 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA21,1)); GmA' Gc_caseA21';];
   T_caseA22 = [eye(size(GmA,1))
zeros(size(GmA,1),size(Gc_caseA22,1)); GmA' Gc_caseA22';];

   v7_caseA6  = inv(T_caseA6' *T_caseA6) *T_caseA6' *[vm7;
zeros(size(Gc_caseA6, 2),1)];
   v7_caseA10 = inv(T_caseA10'*T_caseA10)*T_caseA10'*[vm7;
zeros(size(Gc_caseA10,2),1)];
   v7_caseA13 = inv(T_caseA13'*T_caseA13)*T_caseA13'*[vm7;
zeros(size(Gc_caseA13,2),1)];
   v7_caseA14 = inv(T_caseA14'*T_caseA14)*T_caseA14'*[vm7;
zeros(size(Gc_caseA14,2),1)];
   v7_caseA18 = inv(T_caseA18'*T_caseA18)*T_caseA18'*[vm7;
zeros(size(Gc_caseA18,2),1)];
   v7_caseA21 = inv(T_caseA21'*T_caseA21)*T_caseA21'*[vm7;
zeros(size(Gc_caseA21,2),1)];
   v7_caseA22 = inv(T_caseA22'*T_caseA22)*T_caseA22'*[vm7;
zeros(size(Gc_caseA22,2),1)];


elseif Choice1 == 2

   T_caseB10 = [eye(size(GmB,1))
zeros(size(GmB,1),size(Gc_caseB10,1)); GmB' Gc_caseB10';];
   T_caseB13 = [eye(size(GmB,1))
zeros(size(GmB,1),size(Gc_caseB13,1)); GmB' Gc_caseB13';];
   T_caseB14 = [eye(size(GmB,1))
zeros(size(GmB,1),size(Gc_caseB14,1)); GmB' Gc_caseB14';];

   v8_caseB10 = inv(T_caseB10'*T_caseB10)*T_caseB10'*[vm8;
zeros(size(Gc_caseB10,2),1)];
   v8_caseB13 = inv(T_caseB13'*T_caseB13)*T_caseB13'*[vm8;
zeros(size(Gc_caseB13,2),1)];
   v8_caseB14 = inv(T_caseB14'*T_caseB14)*T_caseB14'*[vm8;
zeros(size(Gc_caseB14,2),1)];


elseif Choice1 == 3
```

```
  T_caseC14 = [eye(size(GmC,1))
zeros(size(GmC,1),size(Gc_caseC14,1)); GmC' Gc_caseC14';];
  T_caseC26 = [eye(size(GmC,1))
zeros(size(GmC,1),size(Gc_caseC26,1)); GmC' Gc_caseC26';];
  T_caseC29 = [eye(size(GmC,1))
zeros(size(GmC,1),size(Gc_caseC29,1)); GmC' Gc_caseC29';];
  T_caseC30 = [eye(size(GmC,1))
zeros(size(GmC,1),size(Gc_caseC30,1)); GmC' Gc_caseC30';];

  v1_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm1;
zeros(size(Gc_caseC14,2),1)];
  v1_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm1;
zeros(size(Gc_caseC26,2),1)];
  v1_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm1;
zeros(size(Gc_caseC29,2),1)];
  v1_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm1;
zeros(size(Gc_caseC30,2),1)];

  v2_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm2;
zeros(size(Gc_caseC14,2),1)];
  v2_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm2;
zeros(size(Gc_caseC26,2),1)];
  v2_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm2;
zeros(size(Gc_caseC29,2),1)];
  v2_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm2;
zeros(size(Gc_caseC30,2),1)];

  v3_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm3;
zeros(size(Gc_caseC14,2),1)];
  v3_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm3;
zeros(size(Gc_caseC26,2),1)];
  v3_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm3;
zeros(size(Gc_caseC29,2),1)];
  v3_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm3;
zeros(size(Gc_caseC30,2),1)];

  v4_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm4;
zeros(size(Gc_caseC14,2),1)];
  v4_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm4;
zeros(size(Gc_caseC26,2),1)];
  v4_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm4;
zeros(size(Gc_caseC29,2),1)];
  v4_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm4;
zeros(size(Gc_caseC30,2),1)];

  v5_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm5;
zeros(size(Gc_caseC14,2),1)];
  v5_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm5;
zeros(size(Gc_caseC26,2),1)];
  v5_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm5;
zeros(size(Gc_caseC29,2),1)];
  v5_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm5;
zeros(size(Gc_caseC30,2),1)];

  v6_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm6;
zeros(size(Gc_caseC14,2),1)];
  v6_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm6;
zeros(size(Gc_caseC26,2),1)];
  v6_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm6;
zeros(size(Gc_caseC29,2),1)];
  v6_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm6;
zeros(size(Gc_caseC30,2),1)];
```

```
  v9_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm9;
zeros(size(Gc_caseC14,2),1)];
  v9_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm9;
zeros(size(Gc_caseC26,2),1)];
  v9_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm9;
zeros(size(Gc_caseC29,2),1)];
  v9_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm9;
zeros(size(Gc_caseC30,2),1)];

  v10_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm10;
zeros(size(Gc_caseC14,2),1)];
  v10_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm10;
zeros(size(Gc_caseC26,2),1)];
  v10_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm10;
zeros(size(Gc_caseC29,2),1)];
  v10_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm10;
zeros(size(Gc_caseC30,2),1)];

  v11_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm11;
zeros(size(Gc_caseC14,2),1)];
  v11_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm11;
zeros(size(Gc_caseC26,2),1)];
  v11_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm11;
zeros(size(Gc_caseC29,2),1)];
  v11_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm11;
zeros(size(Gc_caseC30,2),1)];

  v12_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm12;
zeros(size(Gc_caseC14,2),1)];
  v12_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm12;
zeros(size(Gc_caseC26,2),1)];
  v12_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm12;
zeros(size(Gc_caseC29,2),1)];
  v12_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm12;
zeros(size(Gc_caseC30,2),1)];

  v13_caseC14 = inv(T_caseC14'*T_caseC14)*T_caseC14'*[vm13;
zeros(size(Gc_caseC14,2),1)];
  v13_caseC26 = inv(T_caseC26'*T_caseC26)*T_caseC26'*[vm13;
zeros(size(Gc_caseC26,2),1)];
  v13_caseC29 = inv(T_caseC29'*T_caseC29)*T_caseC29'*[vm13;
zeros(size(Gc_caseC29,2),1)];
  v13_caseC30 = inv(T_caseC30'*T_caseC30)*T_caseC30'*[vm13;
zeros(size(Gc_caseC30,2),1)];

end
```

E.2 analyze_G.m

```
function [rank_Gc,cond_Gc,flux_sens] = analyze_G(i,Gc,Gm)

if det(Gc*Gc') ~= 0
  sprintf('for case %d \n',i);
  rank_Gc = 'full rank';
  cond_Gc = cond(Gc');
  flux_sens = - pinv(Gc')*(Gm');
end
```

Biography

Born in September, 1979, Nuttawut Laksanapanyakul has been raised as the third child in his family that settled down in Bangkok. At Jindamanee school, Bangkok, he could be literate for the first time in 1983. Then he continued his study in Suankularb Witthayalai, Bangkok and graduated a five-year program in 1985. Instantly, he headed to the Faculty of Engineering, Chulalongkorn University, Bangkok for completing Bachelor Degree in chemical engineering and finally graduated in 1999. Without hesitation, he was intent on graduating a Master Degree in chemical engineering majoring in biochemical engineering. As soon as this thesis was approved in April, 2003, he utterly had his hopes fulfilled.