國 立 交 通 大 學

生 物 資 訊 研 究 所

碩 士 論 文

大腸桿菌在無氧環境下利用甘油生產酒精的模擬與分析

Modeling and Analysis of Glycerol Anaerobic Utilization by Escherichia coli

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中 華 民 國 九 十 八 年 七 月

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國立交通大學生物資訊研究所碩士班

中文摘要

世界能源危機的意識，使得利用微生物生產生質燃料如生質酒精、生質柴油等，已被廣泛的研究。雖然過去的研究主要探討以微生物分解纖維素的議題，纖維素降解程序仍然困難且繁鎖。相對地，甘油的化學結構簡單且能夠直接利用，而生質柴油生產的過程會產生大量的甘油。因此甘油成爲生產生質燃料的理想材料。也因此，甘油厭氧利用的機制對於有效生成生質燃料與生物質有一定的重要性。

數學模擬應用於生物系統已有顯著的進展，這些模擬系統包含決定代謝反應的流量、預測基因調節作用以及對於細胞行爲的解讀。雙相(two-phase)研究運用基礎流量模式(Elementary Flux Modes, EFMs)與非線性程式(nonlinear programming)分析並模擬大腸桿菌內甘油代謝之厭氧路徑。我們的分析能夠解釋並提供細菌於甘油厭氧代謝時，其生長問題的解決之道。此外，結合酵素動力準則與基礎流量模式能夠預測流量分布與代謝濃度，甚至作爲基因剔除之參考。 這些資訊有助於代謝工程在基因層次之修飾並藉以提高目標物產量。

Modeling and Analysis of Glycerol Anaerobic Utilization by *Escherichia coli*

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Abstract

Using microbes as the machine to produce biofuel such as bioethanol and biodiesel have been widely investigated due to global energy crisis. While considerable attention has been paid in the past on issues related to decompose the cellulose by microbes, the procedure of cellulose degradation remains heavy and complicate. In contrast, the chemical structure of glycerol is simpler and can be used directly. Thus glycerol became an ideal substrate for biofuel generation because a mass of glycerol produced from biodiesel factory. Therefore, mechanism of glycerol anaerobic utilization is important for efficiently biofuel synthesis and biomass growth. Mathematical modeling for biology systems have progressed tremendously, including determination of metabolic fluxes, prediction of gene regulations, and interpretation of cell behaviors. A two-phase study was designed to utilizing elementary flux modes (EFMs) and nonlinear programming for analysis and modeling glycerol metabolic anaerobic pathway in *Escherichia coli.* Our analysis explains and provides solution for the growth problem of bacteria during glycerol anaerobic metabolism. Besides, the model combining enzyme kinetic principle and elementary flux modes (EFMs) could predict the flux distribution and concentration of metabolites even gene deletion, which helps metabolic engineering to modify gene for product optimization.

致謝

首先要感謝我的指導教授黃憲達老師，在我碩士班時，對於研究的啟蒙以及技術的指導，更在平常的相處中學到的不少做人處事的道理，也在我怠惰懶散時給了我很大的動力完成學業最後的口試以及論文，另外也很感謝隔壁應用微生物與生物工程實驗室的曾慶平老師以及實驗室的學生，在我缺乏生物上的知識以及見解時，給了我很多的意見和指教。

當然也要感覺實驗室的學長姐，不僅是在一開始帶領著我踏上研究的軌道，也在遇到困難時在旁幫助，並且督促著我的研究進度，而與整個實驗室的同仁們的相處也非常融洽，興趣也十分相投，包括棒球，籃球，出去玩，吃東西等等，給了我碩士班生活很多的樂趣。

最後，要感謝家人的支持，因為住在家裡，所以許多大小事都麻煩家人，卻又常常不回家，或是夜晚騎車回家，都帶給了家人一些擔心及關心，而如期完成了碩士的學業，也是因為家人讓我無後顧之憂的專心在學校的研究上，而完成碩士班的學業，都是因為大家的幫助，才有今天的我，謝謝大家。

張恆毅　于 交通大學 2009

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Chapter 1 Introduction

1.1 Biological background

1.1.1 World energy crisis

When we concern about the problem in our life, we'll find that the most important source will be exhausted and unable to regenerate in a few years. Because the resources are versatile, it is used as primary energy source.

World energy dependence is mainly depends on limited resource such as coal, oil or natural gas. With the economic progression, the energy demands are increasing in developed country and under-development country. According to the report from International Energy Agency in 2007, the demands are drastically increasing in recent ten years and will keep surging in the following ten to twenty years, see Figure1.1 (World Energy Outlook, 2007). Owing to mass demands and the finite resource, the supply of energy falls short of demands. Figure 1.2 shows the different fuel prices divided by oil prices in relation to the time scale, which implied that oil price is increasing with time in every way (World Energy Outlook, 2007). It is evident that shortage of this limited natural resource results in energy crisis in the world. Fortunately, there are renewable energy generated from nuclear, hydro and biomass. Figure 1.3 shows the renewable energy constitutes about 80% of bio-energy, which was energy derived from biomass (World Energy Outlook, 2003). Thus the bio-energy may provide a way to lessen the world energy crisis.

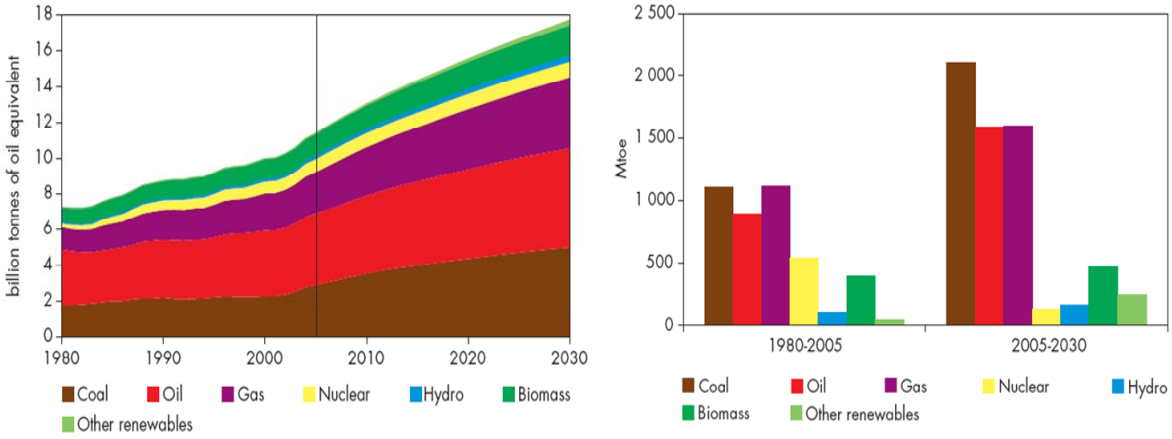


Figure 1.1 World primary energy demand in the reference scenario. (International Energy Agency, 2007)

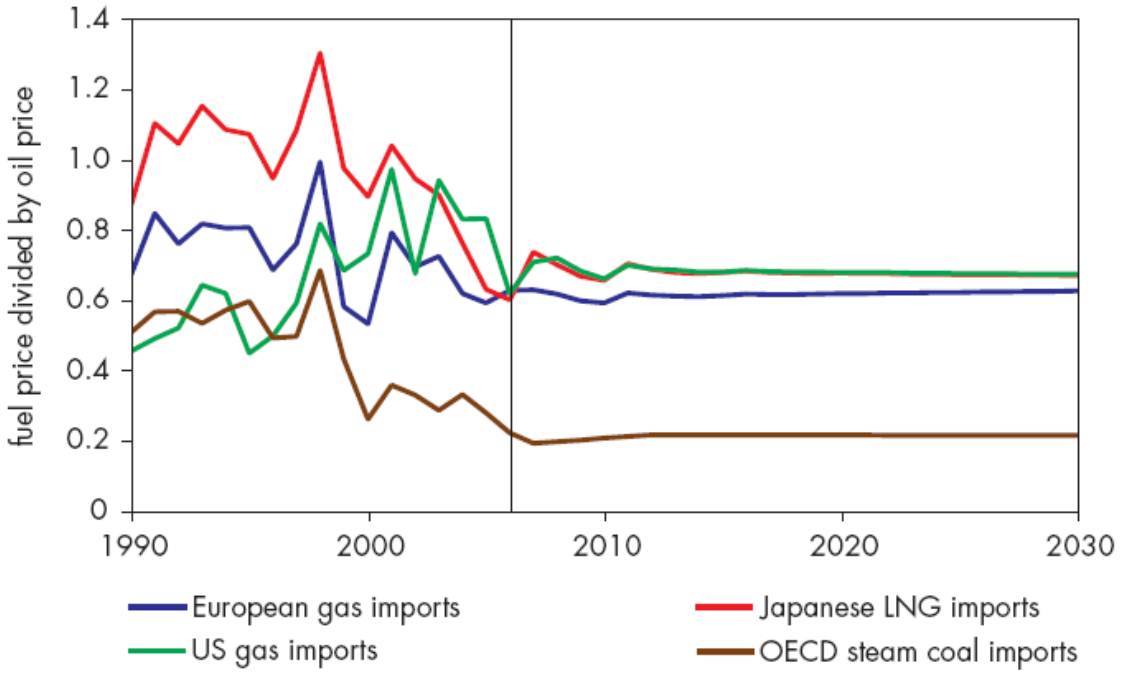


Figure 1.2 Assumed ratio of natural gas and implied relation of coal prices to oil prices to oil prices in the reference scenario.

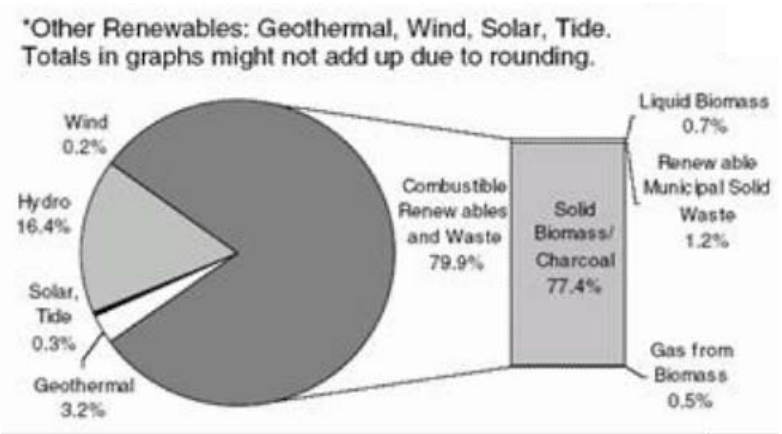


Figure 1.3 Ratio of renewable energy from different sources.

1.1.2 Glycerol for biofuels generation

Biofuels like biodiesel and bioethanol become a biological solution for generating renewable energy, which convert animal or vegetable oil to useful chemical compound biodiesel. The chemical structure and the procedure of biodiesel synthesis are shown at Figure 1.4. During the biodiesel production, byproduct glycerol also produced. The glycerol produced from biodiesel production became competitive compared with those generated from general glycerol factory.

Glycerol is commonly called glycerine or glycerin which was used as materials for flexible foams, serves as humectants, and as a thickening agent in liqueurs. Since 2004, the price of glycerol was cut down dramatically for biodiesel expand vigorously that shown in Figure 1.5. Overproduction of glycerol changed the strategy of glycerol factory such as P&G, Uniqema, Dow Chemical and Cognis. Therefore, glycerol was developed for additional role like sources of hydrogen gas [1] or convert to ethanol [2] for saving the energy crisis.

Previous study showed a lot of research works have been done in application of cellulose as carbon source to biofuels. But only a few recent efforts have focus on glycerol as microbial carbon source. Compare with cellulose, glycerol economizes is not only the work of degradation into small molecular but also cost of operation that shown in Figure 1.6. [3]

Because of its availability, low prices, and high degree of reduction[2], glycerol become a good resource from biodiesel waste. The biofuels second generation aims to improve the efficiency of renewable energy production.

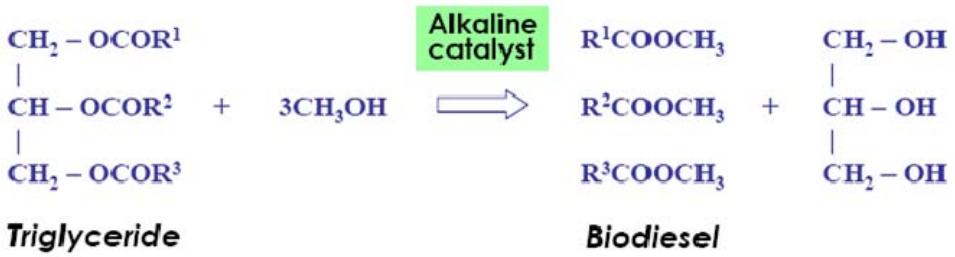


Figure 1.4 Biodiesel production and its byproduct glycerol.

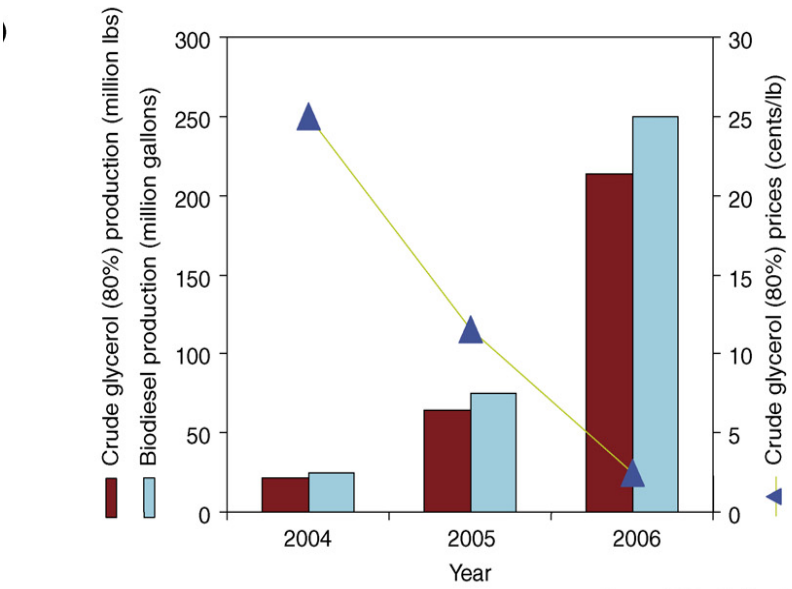
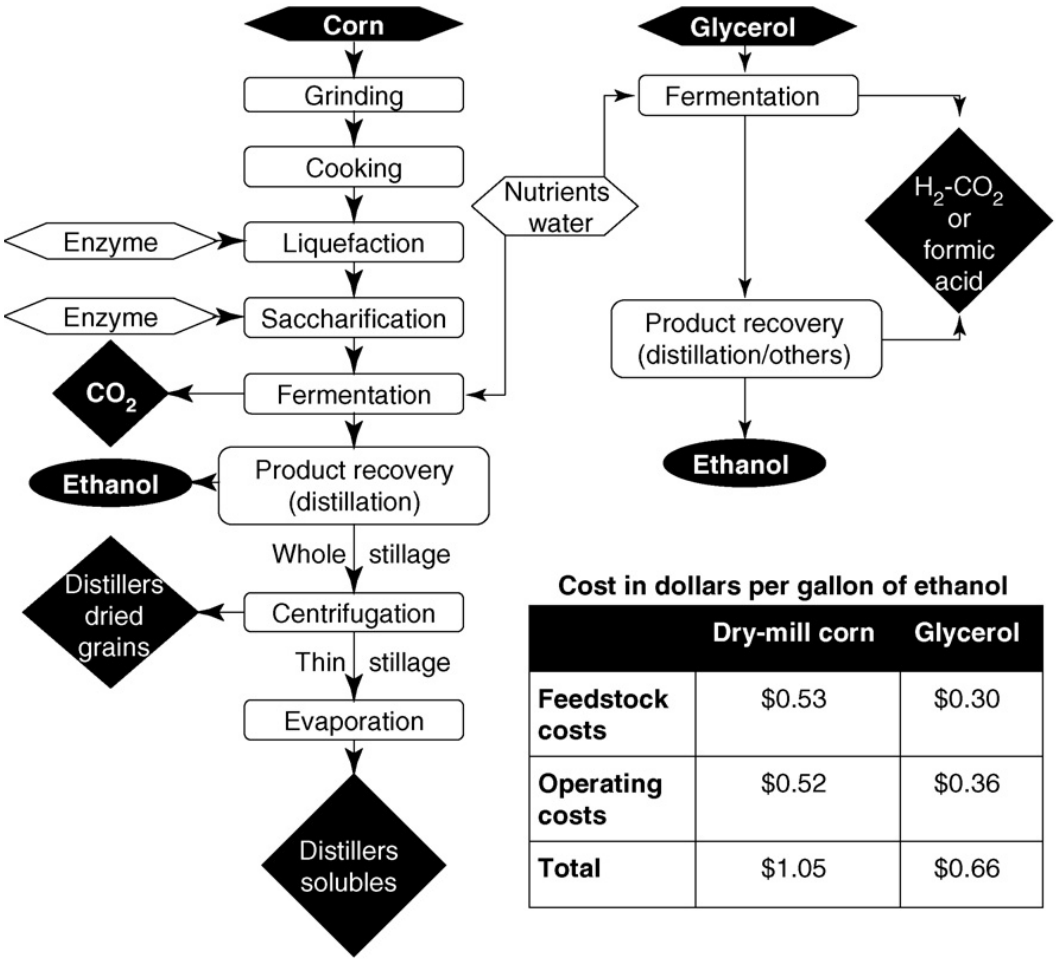


Figure 1.5 US biodiesel production and its impact on crude glycerol prices [2]



Cost in dollars per gallon of ethanol

	Dry-mill corn	Glycerol
Feedstock costs	$0.53	$0.30
Operating costs	$0.52	$0.36
Total	$1.05	$0.66

Figure 1.6 Comparison of ethanol production from corn-derived sugars[2]

1.1.3 Microbes utilize glycerol

The microbes which using glycerol as carbon source in anaerobic condition have some characteristics in gene coding enzymes that utilizing glycerol[4]. That showed the capability of glycerol fermentation are related with 1,3-propanediol dehydrogenase and glycerol dehydratase. But the pathway of glycerol utilize not only use there two reaction that shown in Table 1.1, but also have two path that convert glycerol to glycolysis intermediates for growth biomass and produce fermentation product that shown in Table1.2.they can convert glycerol to 1,3-propanediol, but the yield of 1,3-propanediol from glycerol is not 100%.becuase of NADH and NAD concentration spend affected the reactions are reduction or oxidation . Therefore, glycerol passed through different reactions to achieve chemical and redox potential balance.

Contrast of their pathogenicity and application shown in Table 1.3, *Klebsiella pneumoniae, Clostridium butyricum, Citrobacter freundii and Enterobacter gergoviae* possess intense pathogenicity, *Lactobacillus reuteri* is better for producing the antibiotic. Because of them, the safely and more feasible for gene modify microbe, *Escherichia coli,* that can ferment glycerol in special condition is suitable to be a biofuels synthesizer and recombinant host.

Table 1.1 Capability of glycerol fermentation and distribution of glyDH and 1,3PD-DH in enterbacterial species.

	Glycerol fermentation	1,3-PD dehydrogenase	Glycerol dehydratase
Citrobacter braakii	YES	YES	YES
Citrobacter farmeri			
Citrobacter freundii			
Citrobacter werlamanii			
Enterobacter gergoviae	YES	YES	YES
Klebsiella pneumoniae	YES	YES	YES
Clostridium pasteurianum	YES	YES	YES

Table 1.2 Glycerol utilization pathway reactions.

Glycerol utilization pathway reactions	Aerobic	Anaerobic(have electron acceptor)	Fermentation
glycerol→sn-glycerol-3-p→DHAP→glycolytic intermediates	YES	YES	NO
glycerol→DHA→DHAP→glycolytic intermediates	NO	NO	YES
glycerol→3-HPA→DHAP→,3-propanediol	YES	YES	YES

Table 1.3 Microbial pathogenicity and applications which can ferment glycerol.

Species	Pathogenicity	Applications
Klebsiella pneumoniae	Pulmonary disease, enteric pathogenicity, nasal mucosa atrophy, and rhinoscleroma	Lactose fermenting, facultative anaerobic
Clostridium butyricum	Botulism, tetanus and gas gangrene	Toxic chemicals and detergents
Lactobacillus reuteri		Anti-microbial agent
Citrobacter freundii	In clinical specimens as an opportunistic or secondary pathogen	Ability to convert tryptophan to indole, ferment lactose, and utilize malonate
Enterobacter gergoviae	Nosocomial (hospital-acquired) urinary tract infections	

1.2 Motivation

In the past, considerable attentions have been paid on issues related to decompose the cellulose by microbes. However, the procedure of cellulose degradation is complex and inefficient. Since biodiesel became more and more popular, the major byproduct during biodiesel production, glycerol, also produced with a large amount. Unlike cellulose, the structure of glycerol is more ordinary and can be used directly. Therefore glycerol came out to be an ideal substrate to generate biofuels.

There are many studies on glycerol fermentation in *Escherichia coli,* previous study described that glycerol was not fermented in the absent of external electron acceptor. However, Dharmadi [3] proposed a framework for glycerol fermentation by *Escherichia coli* which showed using tryptone could affect cell growth, yet tryptone is not electron acceptor and the

mechanism of its effectiveness remains unknown.

Recently, the metabolic engineering began to involve with metabolic pathways and gene networks to optimize the yield of metabolites required. However, efforts in experiments to find the beneficial gene for production are too heavy and complicated to execute. Therefore, we need the mathematical method, especially modeling, to facilitate the metabolic engineering implementation. Constructing models using the experimental data combined with chemical and physical knowledge could simulate the behavior of cell and even economize on complicated experiments by reasonable gene selection. Besides, studies on gene expression and enzyme activity dominating metabolites synthesis usually focus on only one enzyme or metabolites, which may overlook the complexity within a cell. Systematic analysis has gathered great importance in recent years. Integrating all its aspects into glycerol anaerobic utilization by *Escherichia coli* could verify the results more correctly.

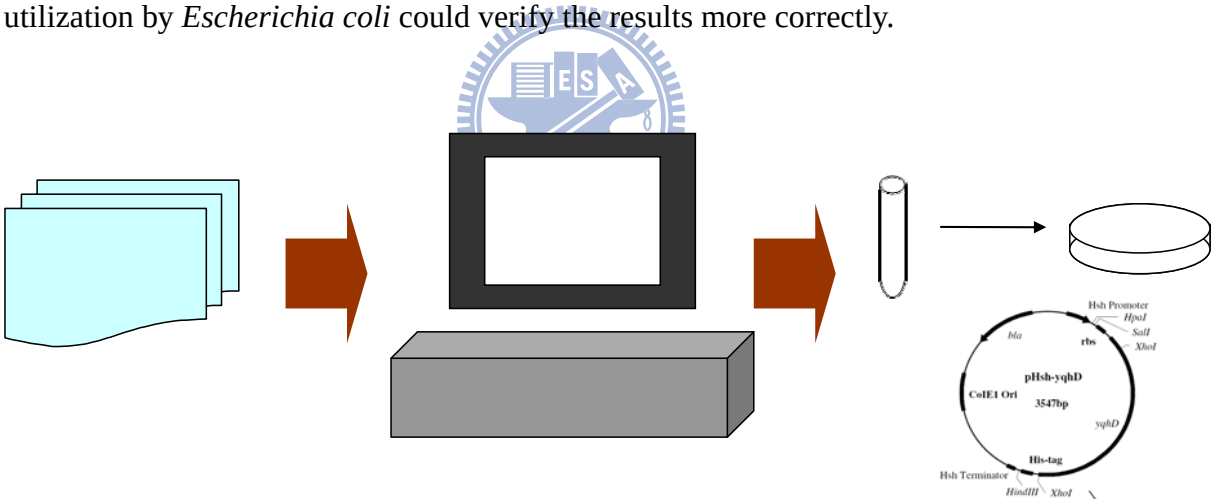


Figure 1.7 systems flow of Modeling and Analysis of Glycerol Anaerobic Utilization by *Escherichia coli*

1.3 Research goals

The purpose of this study was to investigate the mechanism of glycerol anaerobic utilizing in *Escherichia coli*, computational anticipation of glycerol anaerobic utilization, and exquisite verification in simulation from model we construct.

1.3.1 Mechanism of glycerol anaerobic utilizing in *Escherichia coli*

The specific research question in this study addressed concerns on why *Escherichia coli* couldn't immediately ferment glycerol in anaerobic condition and how to solve the problem with different strategies such as replenish tryptone, fumarate, or foreign gene transformation. The methods in previous projects for different objective exists some disadvantages when producing ethanol. Besides, the purpose of those experiments is not for ethanol production. However, the strategies have been proved by many literatures that they actually work in *Escherichia coli.*

Elementary flux modes (EFM) analysis was used to count the possible way of metabolic systems in the metabolic analysis of central carbon. The EFM analysis obeyed the physiological rule, such as the law of conservation of mass. Using elementary flux modes (EFM) analysis can indicate the different condition of possible routes from the external carbon source to the end product. Compared with high yield modes, low yield modes can clearly illustrate the relation between metabolic flux distribution and products yield.

1.3.2 Computational prediction of glycerol anaerobic utilization

The next part of the analysis used hybrid model to extend elementary flux modes usability and can be differ from previous study about elementary flux modes. Previous studies calculate elementary flux modes to represent the whole systems reaction flux distribution, in which each mode will multiply one independent parameter. This did not express metabolic systems characteristics such as the fluctuation of time scale dependant metabolites concentration. The hybrid model methods not only contain the time dependant parameters but also reveal enzyme kinetic based knowledge like Michaelis–Menten kinetics [5].

The parameters have to fit experimental data to construct the computational model that can simulate the metabolic flux and metabolites concentration. If the simulation data

compared with general data are similar, it can be told that parameters fitted correctly and reflected the real experiment.

1.3.3 Verification of simulation derived from constructed model

The model accuracy is of importance when used to predict cell behavior in real system. The process of verifying the model makes it reliable with biologist. When models are used to evaluate strategies in experiments, the results of evaluation is usually made to a fundamental model representing a cellular system, from which systems could be modified and work.

Sensitivity of parameters quantified the correlation between parameters and model's variables. The significance of parameters affecting whole systems can be found. Besides, the parameters distribution also implied the tendency of model system. For example, the specific reaction became momentous role when condition changed. The analysis of parameters accompanied with the evidence of gene modification experiments together demonstrate the anaerobic condition of *Escherichia coli* system utilizing glycerol is an authentic way when predicting the work of *Escherichia coli* behaviors.

Chapter 2 Pathway investigation of glycerol anaerobic utilization by *Escherichia coli*

2.1 Introduction

2.1.1 Glycerol related reaction in Escherichia coli

Using glycerol for generating biofuels is a new strategy differs from cellulose as carbon source in *Escherichia coli* last three years. The major variation of these two carbon source is the reactions of convert substrate to glycolytic intermediate. Glycerol was through glycerol kinase or glycerol dehydrogenase in different conditions. When the environment presents electron acceptor, glycerol converted to sn-glycerol -3-phosphate. After then, sn-glycerol -3-phosphate transferred to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase, which only express in anaerobic condition shown in Table 2.1.

Previous study showed glycerol fermentation can not take place in *Escherichia coli.* *Escherichia coli* grow under anaerobic conditions in a mixture of glycerol together with nitrate or fumarate. However, recent research [3] showed that *Escherichia coli* undergo glycerol fermentation when tryptone added and they prove that tryptone is not electron acceptor by NMR spectra. Yet if tryptone is absent, *Escherichia coli* can not grow in this condition. According to these studies, we proposed that the tryptone is taken as a cell's biomass growing source, from which some redox compounds was provided to push metabolic pathway for glycerol fermentation.

Table 2.1 Enzymes related glycerol utilization in *Escherichia coli*

Condition	Glycerol kinase	Glycerol dehydrogenase	Glycerol-3-phosphate dehydrogenase(GlpABC)	Glycerol-3-phosphate dehydrogenase(GlpD)
Aerobic	YES	NO	NO	YES
Anaerobic(have electron acceptor)	YES	NO	YES	NO
Anaerobic(no electron acceptor)	YES	YES	NO	NO

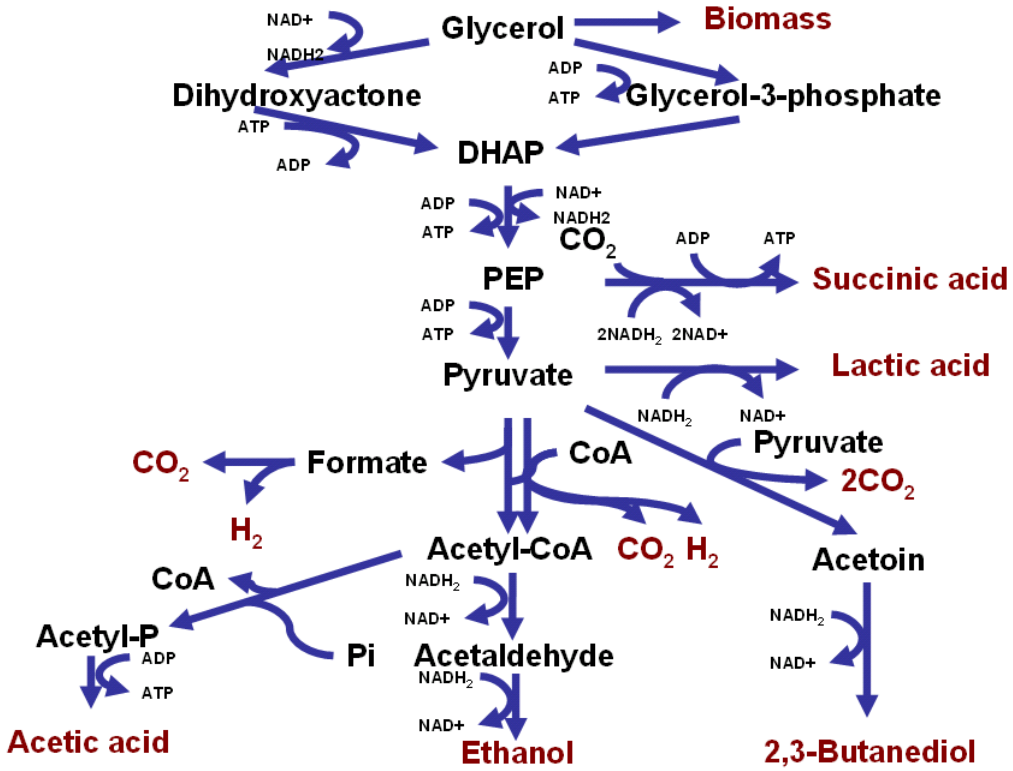


Figure 2.1 Subset of glycerol anaerobic utilization pathway in Escherichia coli.

2.1.2 Elementary flux modes

Because of detailed investigation of genome and enzyme in *Escherichia coli*, the reactions identified and associated with whole cell metabolic pathways responsible for growth

and survival[6]. When intracellular substrates and enzymes presented under suitable condition, the enzyme catalyzed reaction is not restricted by simple decisions. Few routes in metabolic pathway couldn't represent the whole metabolic pathway. Leiser and Blum [7] proposed the "fundamental modes "can be decomposed to a linear structure as a model of elementary flux modes. In a biochemical reaction systems distinguish between border reactions and internal reactions. Border reactions and internal reactions can be distinguished in a biochemical reaction system such as glucose that be feed on *E.coli*, or ethanol flow from *E.coli*. A chemical reaction possess two direct that called the reversible reaction because of enzyme capability, free energy of reactions, and push form the environment. If the reaction is reversible, the numbers of path that elementary mode analyses calculated will be increase. The principle of elementary flux mode is finding the immediate path from substrate to end product, and the numbers of elementary flux mode represent all of possible cell behavior in metabolic pathway. Which elementary flux mode related to different end product in metabolic pathway such as biomass, ethanol, acetate and lactate, is commentated by biological knowledge.

The route from external metabolite go through the direct reactions to the end product shown in Figure 2.1, and every elementary flux modes that is not cyclic have at least one input and one output flux, which allowed multiple compounds in one reaction. The possible, complex pathways in a cell were thought to indicate cell flexibility and robustness to adapt with optima fitness to the environmental conditions by integrating the use of preferable pathways. One of the aims for elementary flux modes was to assign anabolic and catabolic costs to make benefits in different environments[8]. Another aim was to reduce the intricate metabolism to a simple linear path with different properties[9].

Besides, there are various extensions of the elementary flux modes such as thermodynamics rules[9], optimal conversion yields[10] and simulation by multiplying an weight matrix [11].

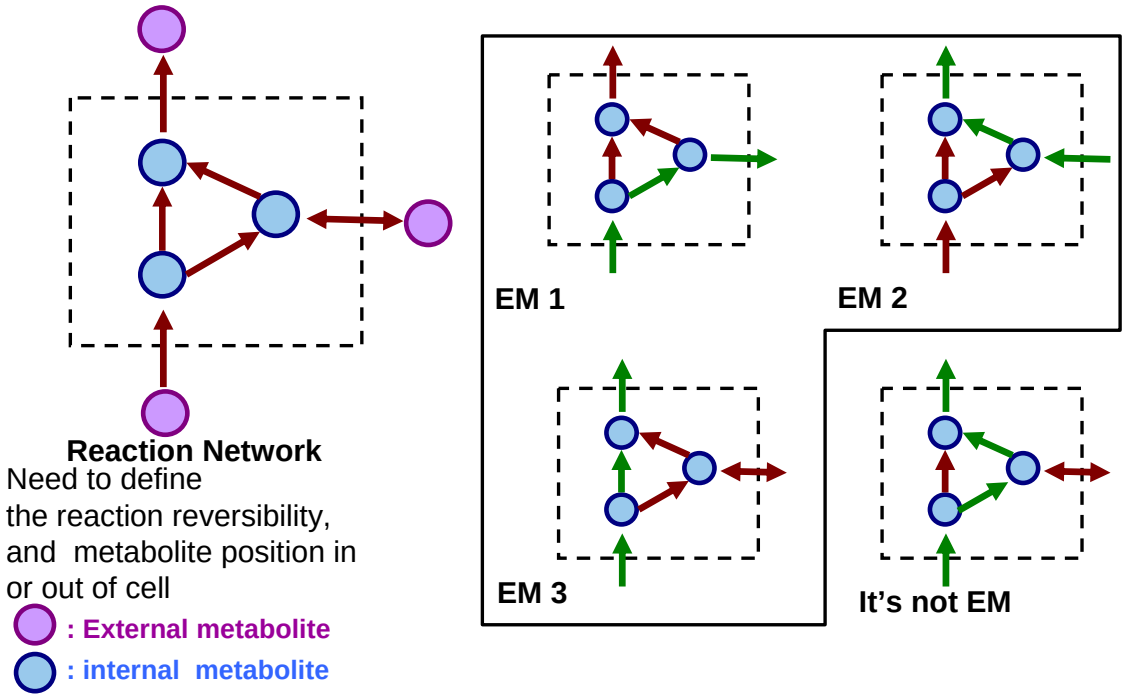


Figure 2.2 Simple example of a biochemical network of elementary flux modes (O.Palsson ,systems biology :properties of reconstructed networks).

2.1.3 Different bacterium glycerol utilize pathway

The reactions of microbes shown in Table 1.1 compare to *Escherichia coli* are something different. Figure 2.3 and Figure 2.4 show major reactions of glycerol utilization in *Escherichia coli* and *Klebsiella pneumoniae.* The reactions in red frame are crucial role for glycerol fermentation, which involves two enzymes as glycerol dehydratase and 1,3-propanediol dehydrogenase. Glycerol dehydratase convert glycerol to 3-hydroxypropionaldehyde and liberate water as electron acceptor that could make up for the lack of electron acceptor in *Escherichia col.* By genetic engineering, these two foreign genes imported from *Klebsiella pneumoniae* to *Escherichia coli* could make the latter one ferment glycerol.

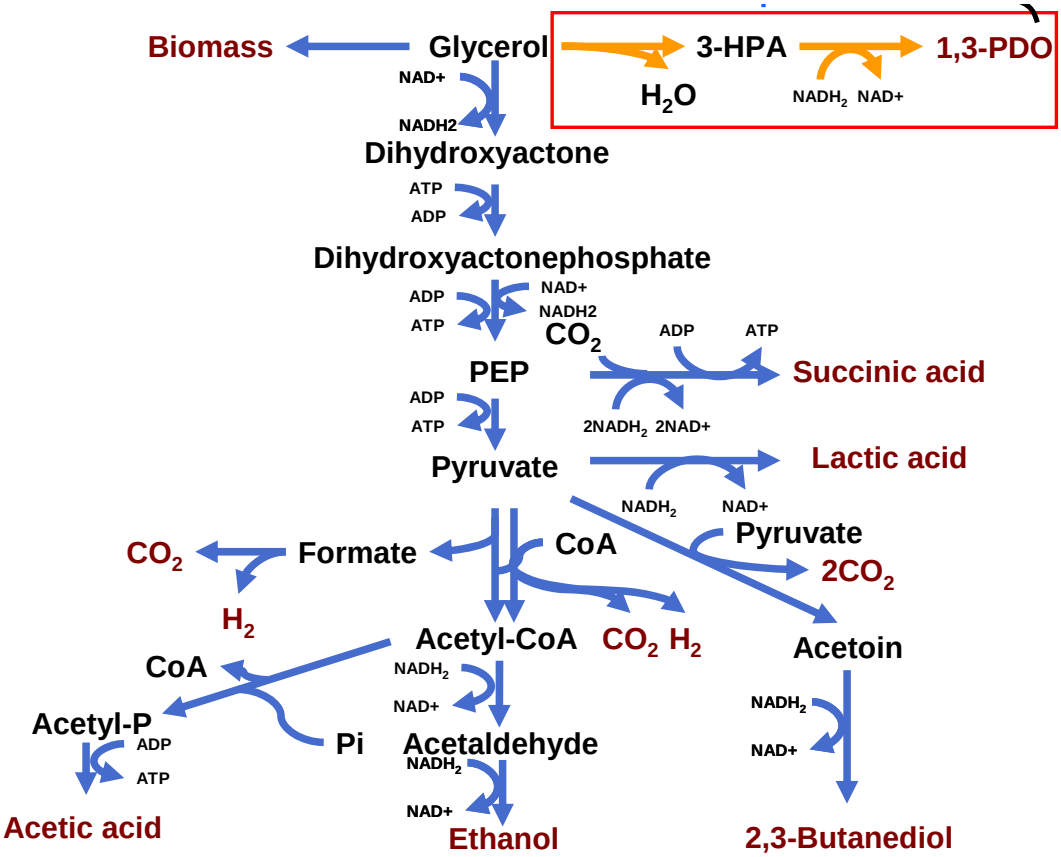


Figure 2.3 Subset of glycerol fermentation pathway in *Klebsiella pneumoniae*. Reactions differ from Escherichia coli with red frame.

2.1.4 Large- scale metabolic network

In microbes, the arrangement of cell is intricate and complicated. Although the key section could explain specific movement, their large-scale structure remains unknown[12].

Trinh's[13] *E. coli* central metabolic network shown in Figure 2.4, which includes glycolysis, gluconeogenesis, pentose phosphate pathway, tricarboxylic acid cycle, fermentative acid pathway, anapleurotic pathway, entner-doudoroff pathway, degradation pathways of pentoses and hexoses, oxidative phosphorylation, maintenance energy, membrane transport, and biomass synthesis. About 70 reactions that can describe major metabolic network in large-scale.

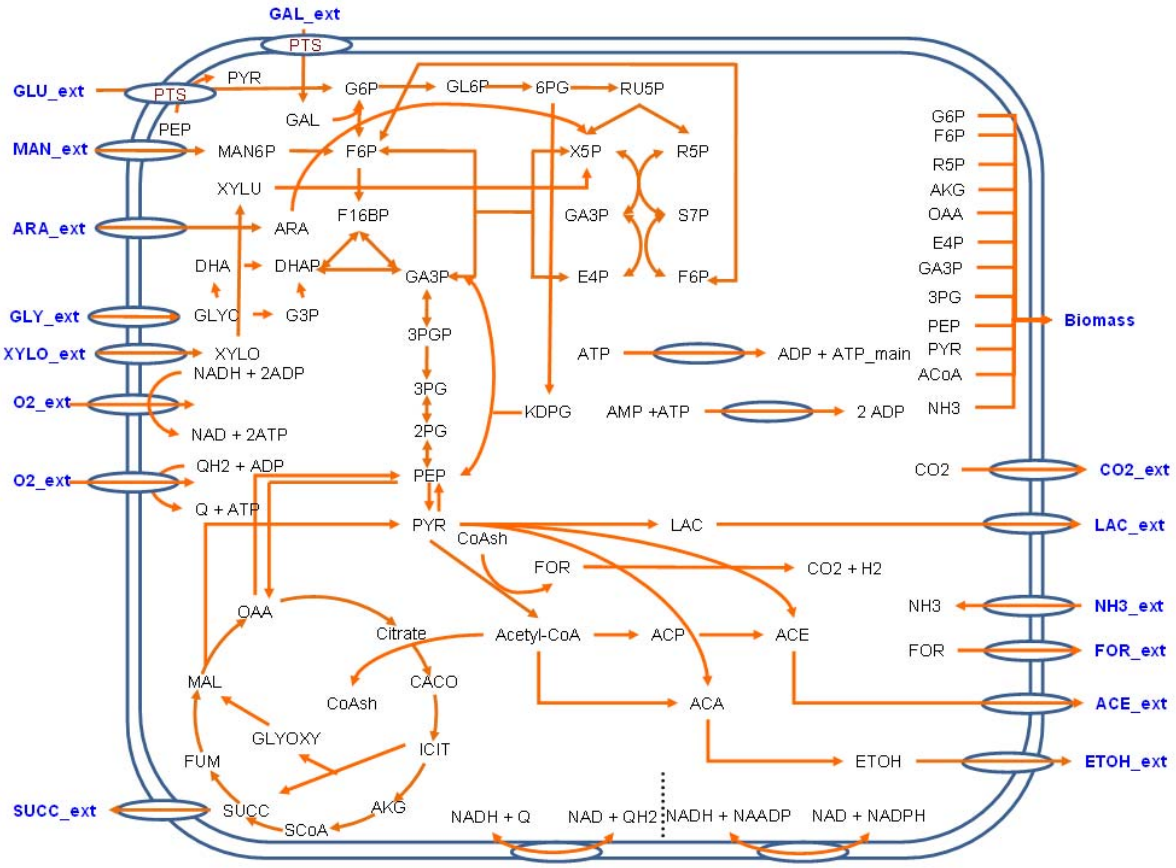


Figure 2.4 Metabolic map of *E. coli* central metabolic network. [13]

2.2 Related work

Early research developed process shown in Table 2.2. At first, researchers proposed that *E.coli* can not ferment glycerol as external electron acceptor to grow unless fumarate of nitrate was used as an exogenous hydrogen acceptor[4].

Later, other species such as *K. pneumoniae, C. butyricum* and *C. freundii* expand glycerol fermentation to 1,3-propanediol, and the major products are 1,3-propanediol and ethanol. Besides, researchers studied on how to improve the yields of 1,3-propanediol. They also found that *K. pneumoniae* couldn't produce 1,3-propanediol only, because of the balance between biomass growth and reduction potential.

Recently, Tong [14] cloned *dha* regulon encoding glycerol dehydratase from *Klebsiella*

pneumoniae to wild-type *Escherichia coli*. They successfully construct a recombinant *E.coli* by importing dha regulon, and found that the growth is not luxuriant. Later, researchers cloned *dhaB* from *Citrobacter freundii* and used it to improve *E.coli* growth[15].

Last three years, Dharmadi used a medium containing high concentration of yeast extracts and tryptone, on which *E.coli* can grow with glycerol under anaerobic condition [3].

Table 2.2 Coordination of glycerol anaerobic utilized literatures.

Condition	Description	Ref.
No growth	Requires electron acceptors	[4]
Plus fumarate	Fumarate, as an exogenous hydrogen acceptor.	[4]
Plus tryptone	Using a medium containing high concentrations of yeast extract and tryptone.	[3]
Other species	Ability to grow fermentatively on glycerol without an exogenous hydrogen acceptor	[16, 17]
Foreign gene express in *E.coli*	Their purpose for clone dha regulon genes to *E.coli* producing 1,3-propanediol	[14, 15, 18]
Plus glucose	Glucose will be uptake first and become major carbon source	

2.3 Motivation and the Specific aim

With previous study on glycerol anaerobic utilization in *Escherichia coli*, we use elementary flux modes analysis to explain why *Escherichia coli* can not grow in glycerol under anaerobic condition. And then we calculate the possible yields in each mode using different strategy for *Escherichia coli* glycerol anaerobic utilization.

2.4 Materials and methods

That calculated all EFMs using METATOOL 5.0, Matlab-based software package for fast and flexible elementary modes analysis. [19]

The METATOOL 5.0 input file consists of three parts. The first one is reactions in the

metabolic network we concerned like Table 2.3. The second is reversibility of enzymes of reactions, express which reaction can react in reverse directly. And the final part is position of metabolite, which described which metabolite was used as end product and initial substrate. To construct three METATOOL file for calculate elementary flux mode, there are glycerol fermentation with tryptone added medium, glycerol anaerobic utilization with fumarate added medium, and glycerol fermentation in *E.coli* which expressed foreign gene dhaB. The knowledge of reactions and enzymes was shown in Table 2.4.

Table 2.3 Example of METATOOL input file‘s reactions.

No.	Reaction
GG1	GLC_external + PEP = G6P + PYR
GG2r	G6P = F6P
GG3	F6P + ATP = F16BP + ADP

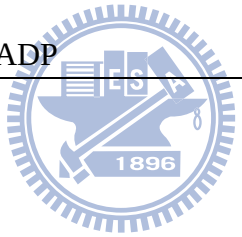


Table 2.4 Pathway data about glycerol anaerobic utilization .

Data description	Ref.
Metabolic map of E. coli central metabolic network	[13]
Glycerol degradation pathway	[3, 6]
1,3-propanediol production pathway	[14, 15]

.

2.5 Results

In this section, explanations of cell growth condition are investigated by elementary flux modes analysis. The ideality yields of each condition are discussed for high yields mode and low yields mode. Finally, the selected mode with both high biomass and high ethanol yields are spread up each reactions flux quotiety.

2.5.1 Numbers of elementary flux modes

Verifying to previous study find that *E.coli* can not directly grow in anaerobic condition that absence external electron acceptor. Compare to other carbon source, numbers of EFMs shown in Table 2.5.the numbers of Glucose is more than the others, obviously *E.coli* had place importance on digest glucose. See the glycerol part , the numbers of EFMs is very few and the EFMs related biomass is zero ,that show *E.coli* can not grow in this condition , it correspond to previous study.

This analysis used EFMs in glycerol fermentation in *E.coli* is directly explain that only glycerol as carbon source would not be utilized to generate biomass. Compare to *E.coli*, *Klebsiella pneumoniae* only add two reactions, but its numbers of EFMs are 1762.

The biological significant of each carbon source in anaerobic condition is present in the numbers of EFMs, which *E.coli* feed on glucose contain 5010 EFMs more than feed on other carbon source ,because of preferable import channel such as phosphoenolpyruvate phosphotransferase system provide more efficiently and more important with carbon source.

Because of the coenzyme NADH and NAD+ are key role of some reactions that include in biomass growing , if NADH had not generate from reactions ,the biomass reactions can not obtain the require compound, that didn’t grow biomass at all.

The different strategies of glycerol anaerobic utilization have a common ground about electrons transformation, first is tryptone provide biomass growing factor: NADH generated to push glycerol dehydrogenase activate, second is fumarate convert to succinate and accept two electrons , third is using foreign enzyme to accept two electrons and effuse water.

Table 2.5 Numbers of elementary flux modes in different carbon source and conditions.

	Xylose or Arabinose	Glucose	Mannose	Galactose	Glycerol	Glycerol in K.pneumoniae
Anaerobic EFMs	1004	5010	2841	1620	18	1762
ETOH	964	4913	2745	1580	18	406
Biomass	443	4157	2134	1297	0	1357
ETOH and Biomass	415	4080	2064	1269	0	326
Ethanol yield	0~0.51	0~0.51	0~0.51	0~0.51	0.25~0.5	0~0.5
Biomass yield	0~0.19	0~0.31	0~0.31	0~0.21	0	0~0.3
Reference	Trinh et al. 2008	Trinh et al. 2008	Trinh et al. 2008	Trinh et al. 2008	This study	This study

Expect the biomass reactions part, the EFMs's overall reaction descript that glycerol fermentation is inclined to produce ethanol shown in Table 2.6.

The preliminary elementary flux modes for three part of glycerol utilization in *E.coli* presented in Table 2.7, that depicted the three condition for *E.coli* are more likely to produce ethanol or grow more biomass such as glycerol + tryptone the ratio of EFMs of biomass/anaerobic are very high, that obviously said grow in glycerol + tryptone exuberant.

Table 2.6 Overall reaction of glycerol fermentation.

EFMs ratio	Overall reaction
6/18	GLYCEROL_ext = H2_ext + ETOH_ext + CO2_ext
6/18	2 GLYCEROL_ext = H2_ext + ETOH_ext + SUCC_ext
6/18	GLYCEROL_ext = ETOH_ext + FOR_ext

Table 2.7 Numbers of elementary flux modes in different strategy for *E.coli* no growth problem

	Glycerol + tryptone	Glycerol + fumarate	Glycerol +1,3 PDO pathway
Anaerobic EFMs	442	1952	1762
Ethanol	114	940	406
Biomass	399	1428	1357

2.5.2 Relationship between yields of biomass and ethanol

That detail concern the yields of each modes, we can see Figure 2.5 (B), that three of blots descript the best ethanol produce mode, best biomass grow mode, and blank mode to compare to others. The modes include what reaction shown in Figure 2.6 ~ Figure 2.8, those reactions different in obviously in Figure 2.6 and Figure 2.7 are Entner-Doudoroff pathway, and different in Figure 2.8 are respiration (anaerobic) pathway and produce acetate acid. It shows a relationship of biomass and Entner-Doudoroff pathway.

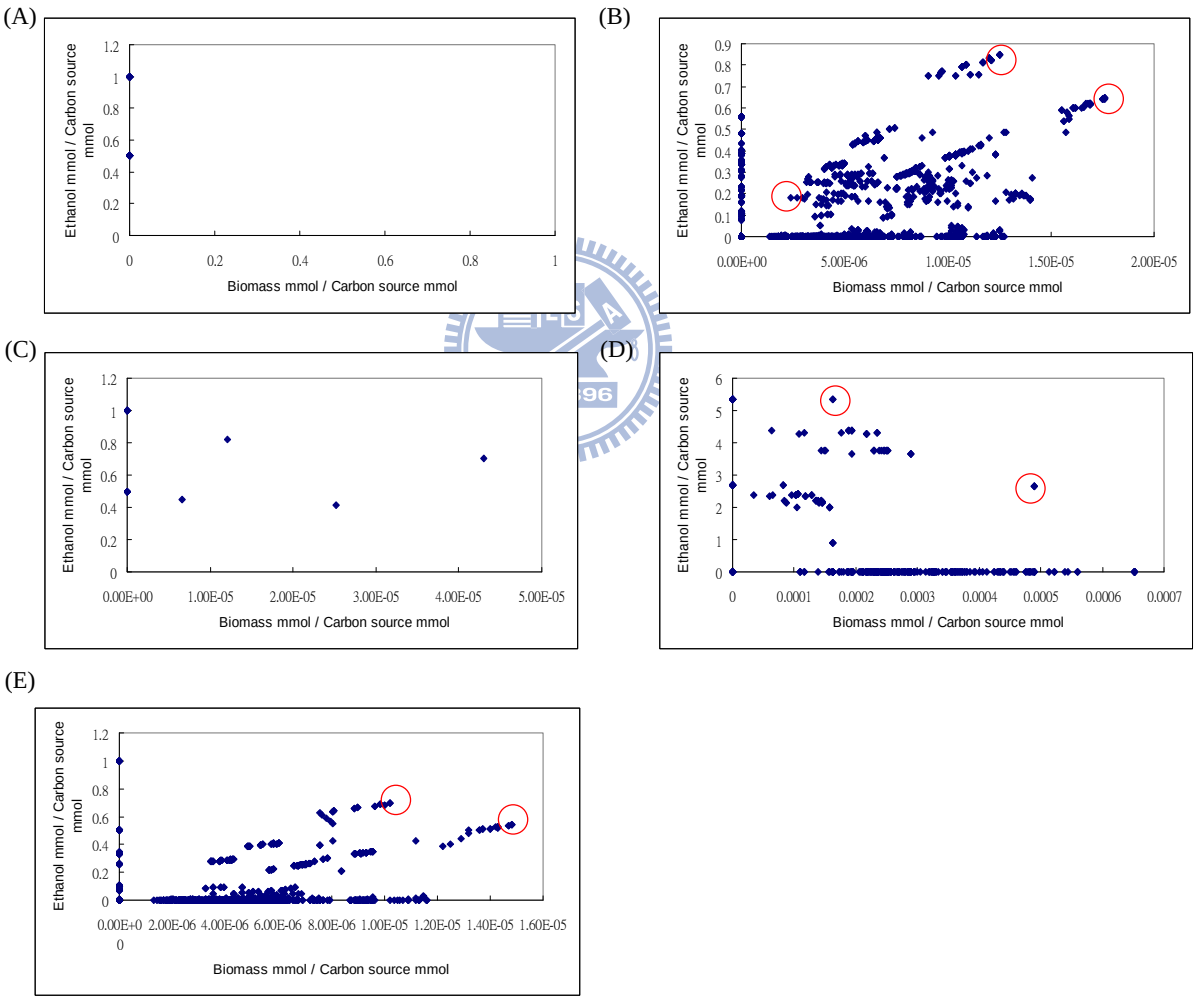


Figure 2.5 Yields of biomass and ethanol (A) Modes of glycerol anaerobic utilization in medium plus fumarate which relate only glycerol (B) Modes of glycerol anaerobic utilization in medium plus fumarate which relate fumarate and glycerol (C) Modes of glycerol fermentation in medium plus tryptone which relate only glycerol (D) Modes of glycerol fermentation in medium plus tryptone which relate glycerol and tryptone (E) Modes of glycerol fermentation in recombinant *E.coli.*

Compare the others condition, the modes of most high ethanol yields and most biomass growing are similar to above conditions. Correspond to our purpose, that Entner-Doudoroff pathway is related with biomass growing.

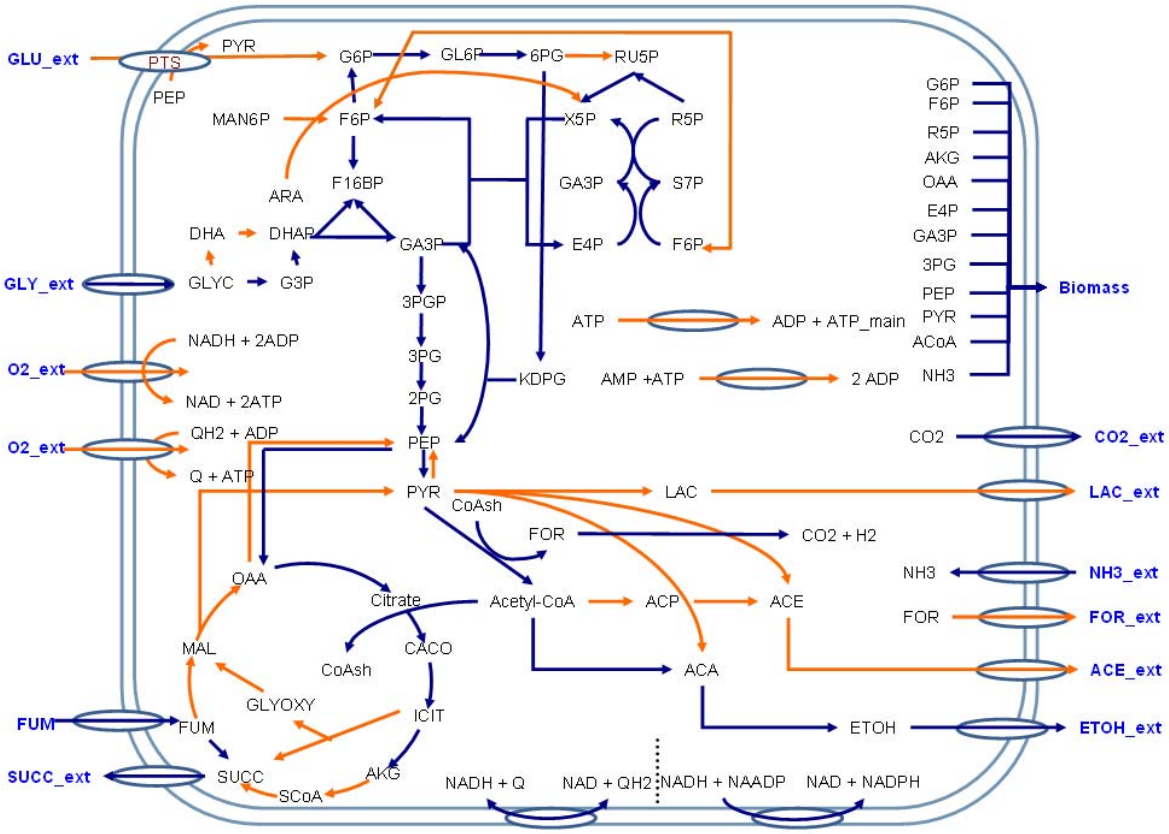


Figure 2.6 The mode of most high yields of ethanol in glycerol anaerobic utilization in medium plus fumarate.

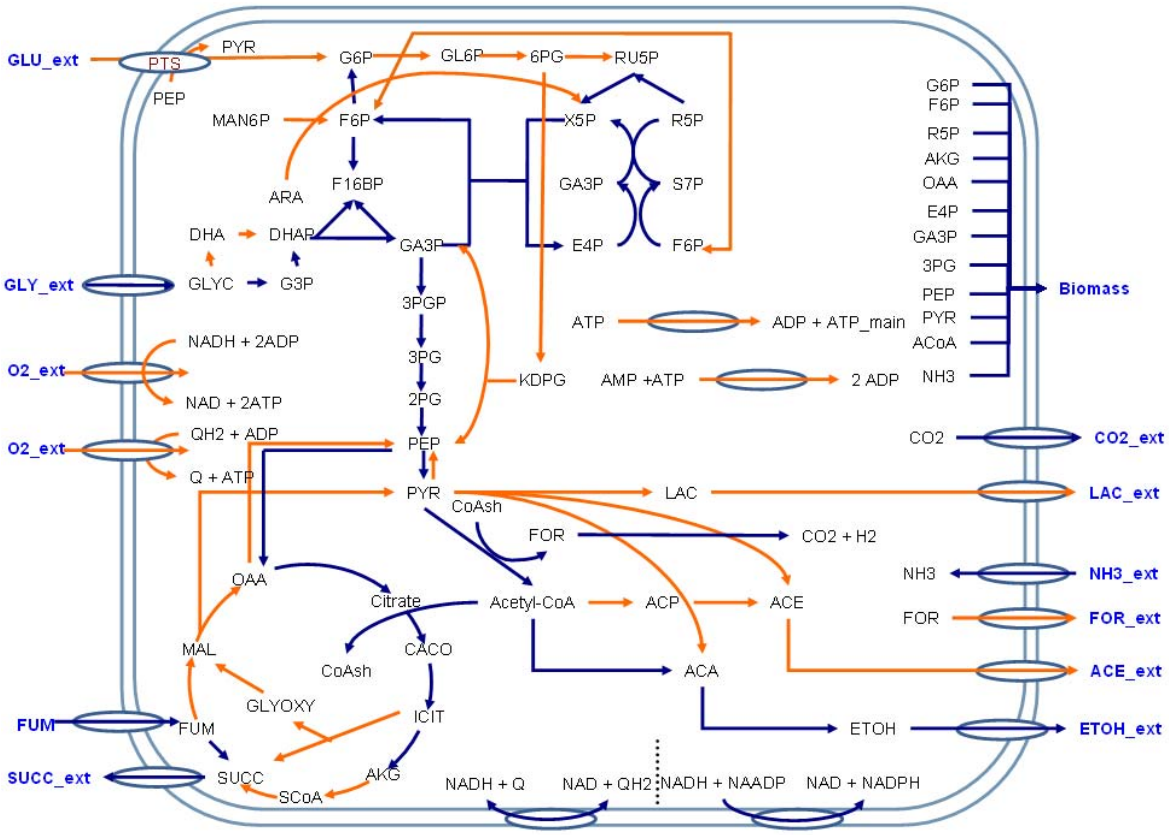


Figure 2.7 The mode of most high yields of biomass in glycerol anaerobic utilization in medium plus fumarate.

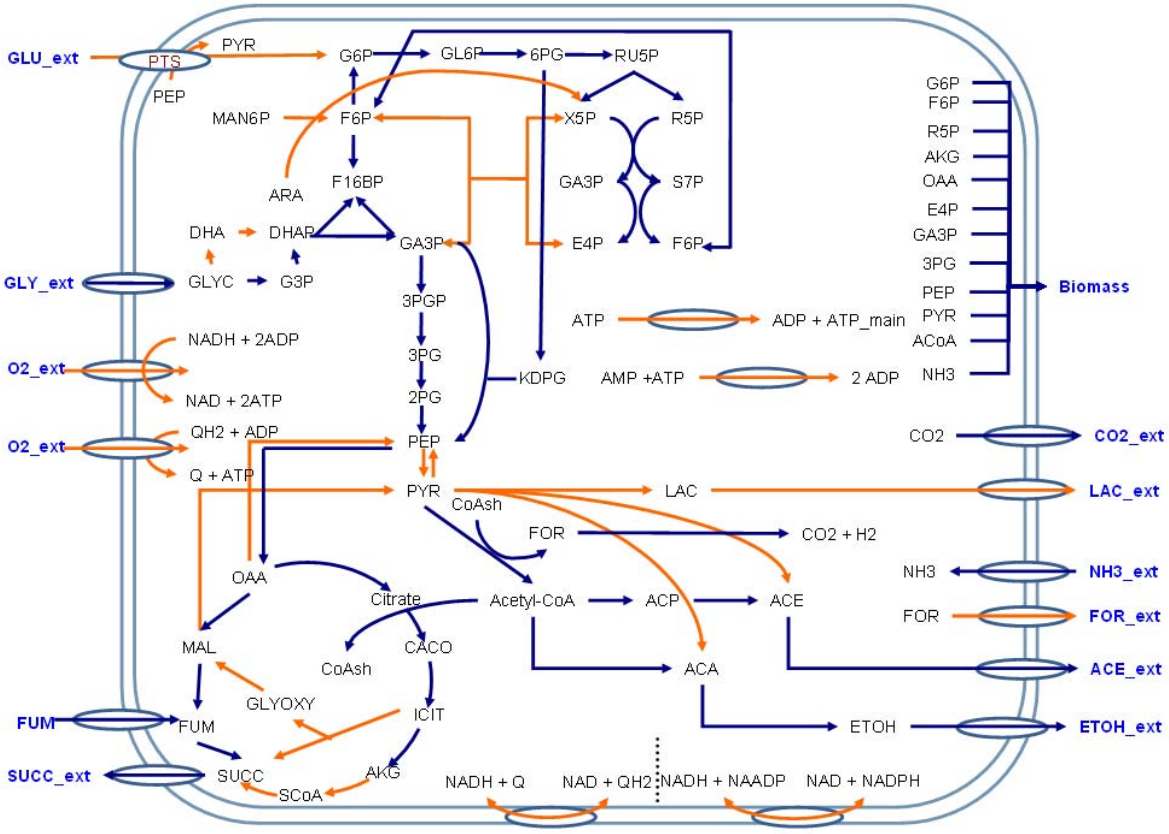


Figure 2.8 The mode of average yields in glycerol anaerobic utilization in medium plus fumarate.

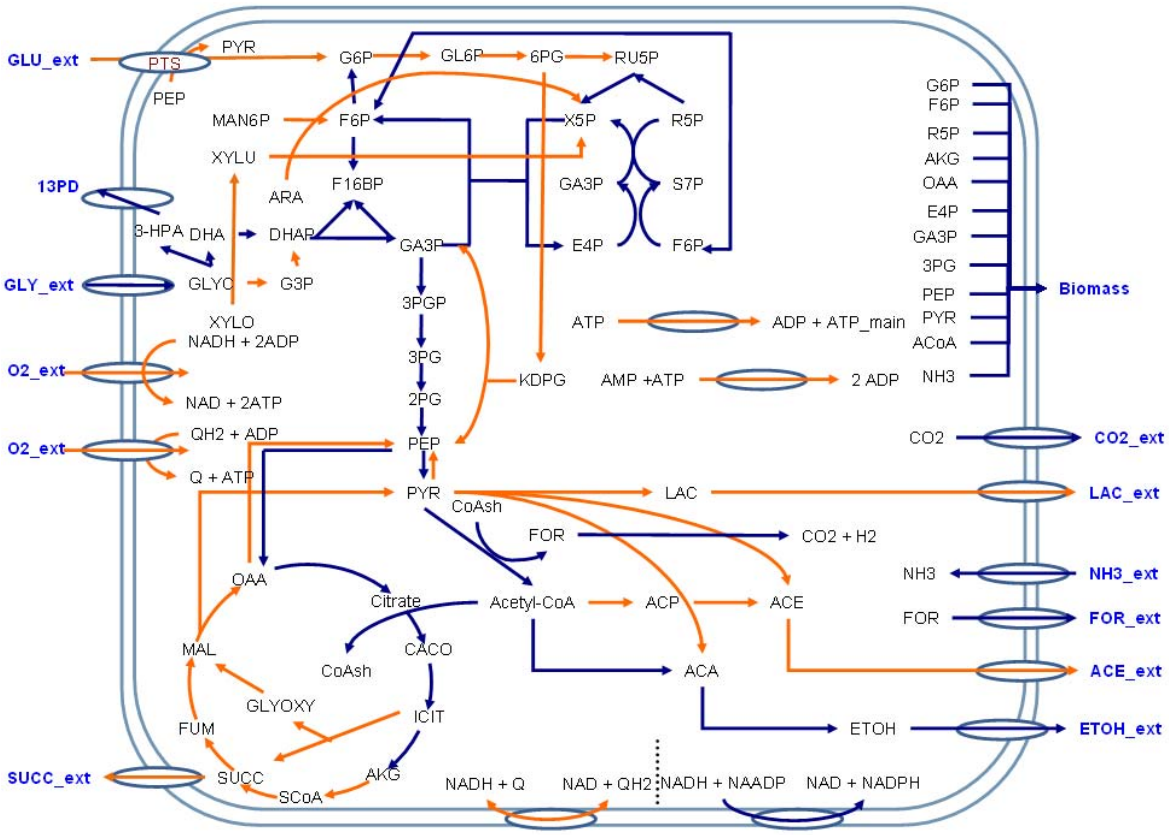


Figure 2.9 The mode of which most high yields of ethanol is in glycerol fermentation express foreign genes.

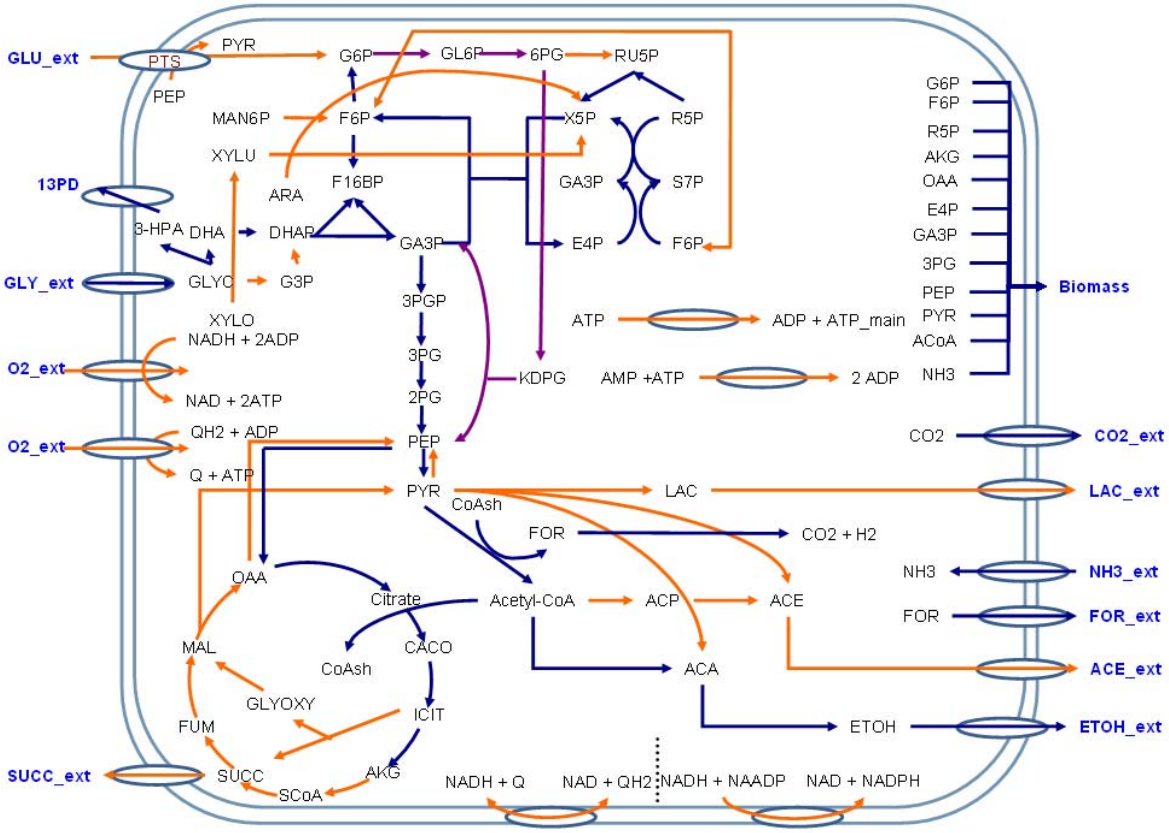


Figure 2.10 The mode of which most high yields of biomass is in glycerol fermentation express foreign genes.

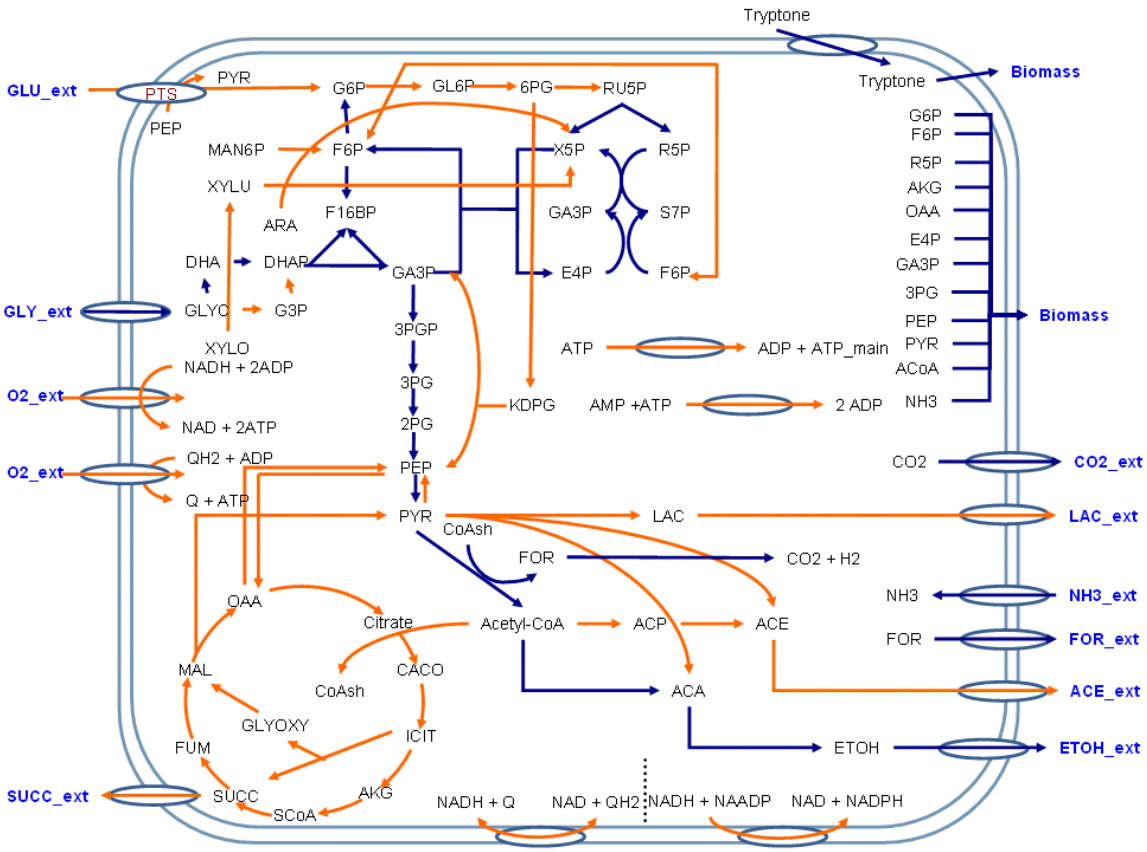


Figure 2.11 The mode of which most high yields of ethanol is in glycerol fermentation in medium plus tryptone.

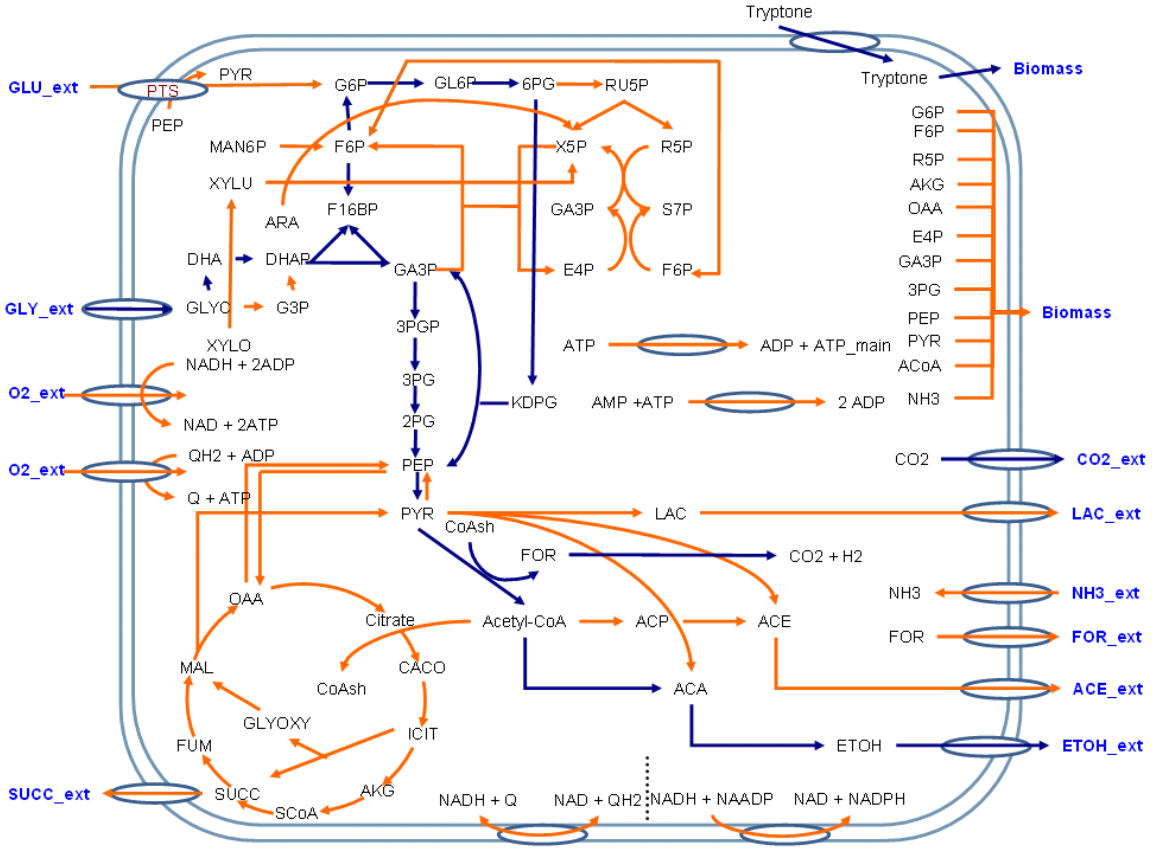


Figure 2.12 The mode of which most high yields of biomass is in glycerol fermentation in medium plus tryptone.

2.6 Summery

The yields of product are important for factory to produce a large amount, investigating possible yields use elementary flux mode that provide valuable information.

Above all, the analysis indicated the reason about *E.coli* glycerol fermentation absence electron acceptor problem, no any biomass growing mode in elementary flux mode analysis, so we can correspond to previous study, and them which reason about biomass no growth may be some redox factor like NADH is scanty, so we find the solution strategy from previous research that consist three part, first is cultivating *E.coli* in medium plus electron acceptor like fumarate , second is cultivating *E.coli* in medium plus tryptone for biomass growing , third is cultivating *E.coli* express foreign gene dhaB from *Klebsiella pneumoniae.*

The analysis of elementary flux mode about the glycerol pathway shown the mold of high yields pathway, and we show the pathway that is most high yields, descript the possible effect in biomass growing and ethanol production.

Chapter 3 Modeling and simulation of glycerol anaerobic utilization behavior in *Escherichia coli*

3.1 Introduction

3.1.1 Modeling for metabolic engineering

Metabolic engineering consist of two parts. One is the development of strategies for control pathways in microbes, and the other use actual biotechnological experiments to complete such strategies [20]. In other words, there are theories and execution involve in metabolic engineering. This study is critically important in considerable decision for implementation of efficient experiments.

Besides, metabolic engineering has a large amount experiment for change expression level of gene, or the different condition for specific enzyme activity. Prediction not only implies the result of experiments, but also explains the biological significant for complex biochemical experiments.

The relationship between substrates in a chemical reaction can be summarized quantitatively by stoichiometry [21]. When breaking metabolic network down into a stoichiometric matrix, the rows and columns in the matrix represent participated chemicals and reactions themselves, respectively. To infer the possibilities from the metabolic network, recent study work on two approaches, that is extreme pathways and elementary mode analysis [22].

3.1.2 Simulation of biological systems

The simulations of biological systems contain three aspects such as gene regulatory network, metabolic pathway, and signal transduction pathway. Three parts represent intracellular behavior fundamental elements that are gene, protein, and metabolite. Large-scale simulations regenerate gene expression and how many genes are regulated, genes possessed translation to protein that will bind to other protein or react with chemical compounds, called protein-protein interaction and metabolic pathway. But the transition of each fundamental element is difficult problem for scaling the heterogeneous data and parameters. Therefore, the research focus on one part of aspect ignored other part's influence.

The usage of simulation are confirming the corresponding of mathematical model with a set of experimental data, predicting the behavior that experiments didn't prove , and the biological significant of cell behaviors[23].

3.2 Related work

3.2.1 Flux balance analysis (FBA)

Flux balance analysis is a different way to simulate the metabolic network using linear programming. There's only single solution resulted from flux balance analysis, which differs from elementary mode analysis and extreme pathways. Because linear programming is usually used to get the maximum potential from the objective function investigated, single solution became ideal for the optimization problem when using flux balance analysis [24].

When approaching flux balance analysis, only metabolites entering or leaving particular reaction in system could be used to exchange fluxes. Those metabolites consumed in the reactions are not assigned any flux value exchanged. Besides, the constraints in exchanged

fluxes along with the enzymes ranging from negative to positive value.

3.2.2 Enzyme kinetic

Enzyme kinetic is research of relation of enzyme and substrate. When substrate bond to enzyme, the protein structure of enzyme were changed and the activity of enzyme responded binding affinity.

The Michaelis–Menten equation relates the initial reaction rate v0 to the substrate concentration. The corresponding graph is a hyperbolic function; the maximum rate is symbolized as Vmax.

The number of reactions per second catalyzed per mole of the enzyme was defined as reaction rate and symbolized as V. According to Michaelis–Menten equation, the reaction rate increases when substrate concentration increasing and the maximum rate may approach to Vmax.

3.3 Motivation and the Specific aim

Simple elementary flux modes is topological analysis of metabolic network, it doesn't realize the cell behavior such as time dependant metabolite concentration. And the simulation of metabolite concentration is vital to the biologist because of cell dynamic change can tell us much valuable information. So we expand the elementary flux modes using hybrid method which Kim[25]develop, correctly simulate time series data similar to experiments.

3.4 Materials and methods

There are result of elementary flux mode analysis, and experimental data from literatures, using the hybrid model that combined EFMs and enzyme kinetic base parameters of metabolic dynamic simulation for glycerol anaerobic utilization in *Escherichia col*i.

3.4.1 Experimental data source

Experimental data were collected from literature, shown in Table3.1, include Glycerol fermentation in medium add tryptone, express foreign gene dhaB from *Klebsiella pneumoniae*, and cultivate in medium which contain fumarate.

Table 3.1 Experimental data source.

Data description	Reference
Glycerol fermentation add tryptone	[26]
Recombinant E.coli which can produce 1,3 PDO	[27]
Glycerol anaerobic utilize add fumarate	[28, 29] [30]

3.4.2 Method of hybrid model

Kim and his group [25] used the elementary flux mode decomposition to express the reaction rate vector by

$$\mathbf{Z}^{\mathrm{T}}\mathbf{r}_{\mathbf{M}} = \mathbf{r}$$

Z is the matrix represents all of elementary flux modes. For example, when we have 4 reactions that contain 8 elementary flux modes, Z is a 4 X 8 matrix. then r_M is each EFMs regulated uptake rate vector, that represent each of EFMs multiply regulated uptake rate vector will get each reactions rate depend times. And metabolite concentration can be calculated by each reaction rate.

r_M defined in Figure 3.1,that show r_M is similar to enzyme kinetic model: Michaelis–Menten kinetics [5].

$$v_0 = \frac{v_{\max}[S]}{K_M + [S]}$$

K_i is the saturation constant, e_i is the enzyme level for elementary flux mode, and k_{max} is maximum uptake rate of elementary flux mode. Detailed explain shown in Figure 3.1, first we got the EFMs like matrix contain amount of EFMs and the reactions in this network, second we generated transposed matrix and use initial parameters for calculate the regulated flux vector r_M, third carried matrix multiplication out .finally we can get the reaction rate in procession .Reaction rate provide how fast of substrate transform to product, that were influenced by temperature, pH value, cofactor, inhibitor and other environment variables. The end product concentration can be calculated by reactions rate of each reactions. For example, the reaction rate V_0 that dependant times, and the product B initial concentration x. when next time step B concentration is x +V_0.

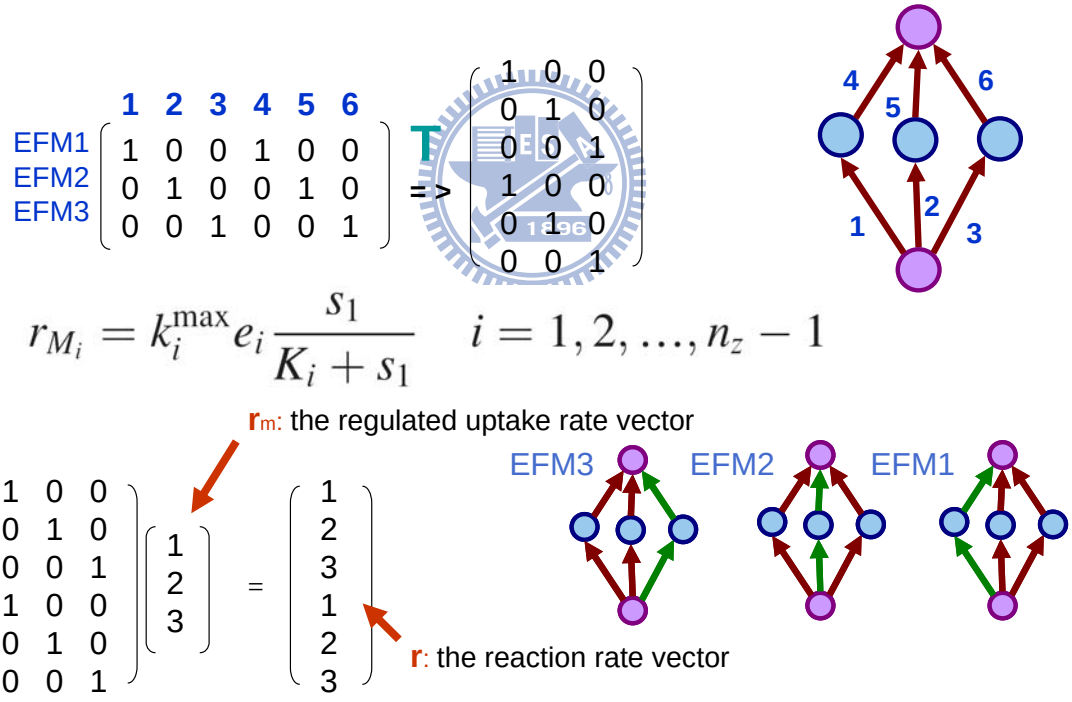


Figure 3.1 Proceed of hybrid model simulated reactions rates.

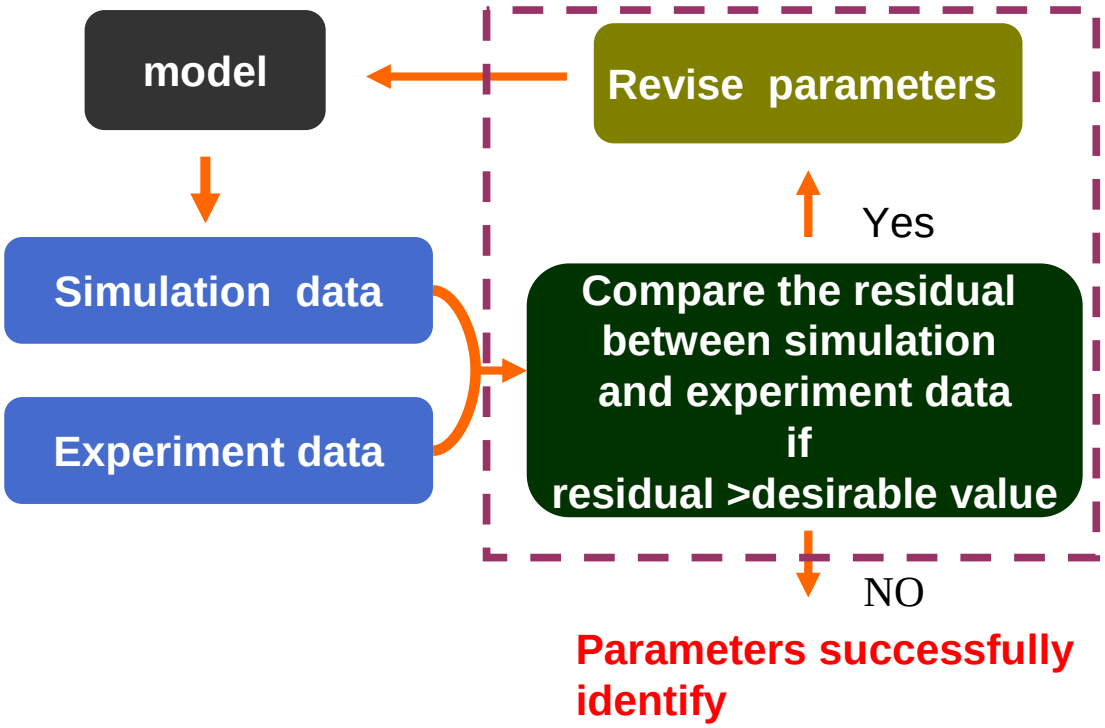


Figure 3.2 Schema of parameters identification.

But this result of simulation is worse because of parameters didn't match with experimental data. Therefore, we have to train the parameters of model shown in Figure 3.2, because of the parameters involve in the kinetic base, that need the solver for nonlinear least square problem, using Tomlab ,matlab package software that is powerful optimization platform and modeling language for solving applied optimization problems in Matlab.

The training flow need a threshold residual for confirm the parameters quality, the residual represent the distance different with simulation and experimental data. If residual value bigger than threshold value, parameters will alter for decrease residual. When parameters smaller than threshold value, that we can called the parameters successfully identify.

3.5 Results

The results show experimental data in different conditions that contain of metabolite concentration disputant times, which include formate, succinate, glycerol, fumarate, biomass, lactate, and ethanol. Figure 3.3 ,Figure3.5 and Figure3.7 illustrate the experimental data from literature that rough descript the yields of each metabolite .and them Figure 3.4 ,Figure3.6 and Figure3.8 depicts the comparisons of metabolite experimental data and simulation data from our model generated, which the variation of predict data and real data was small when the metabolite concentration is not too small to calculate, and see the Figure 3.4 (B), the variation seem very large , but the real variation compare with others is very small.

In this study, the model construction fit to experimental data was successful to simulate the quality data.

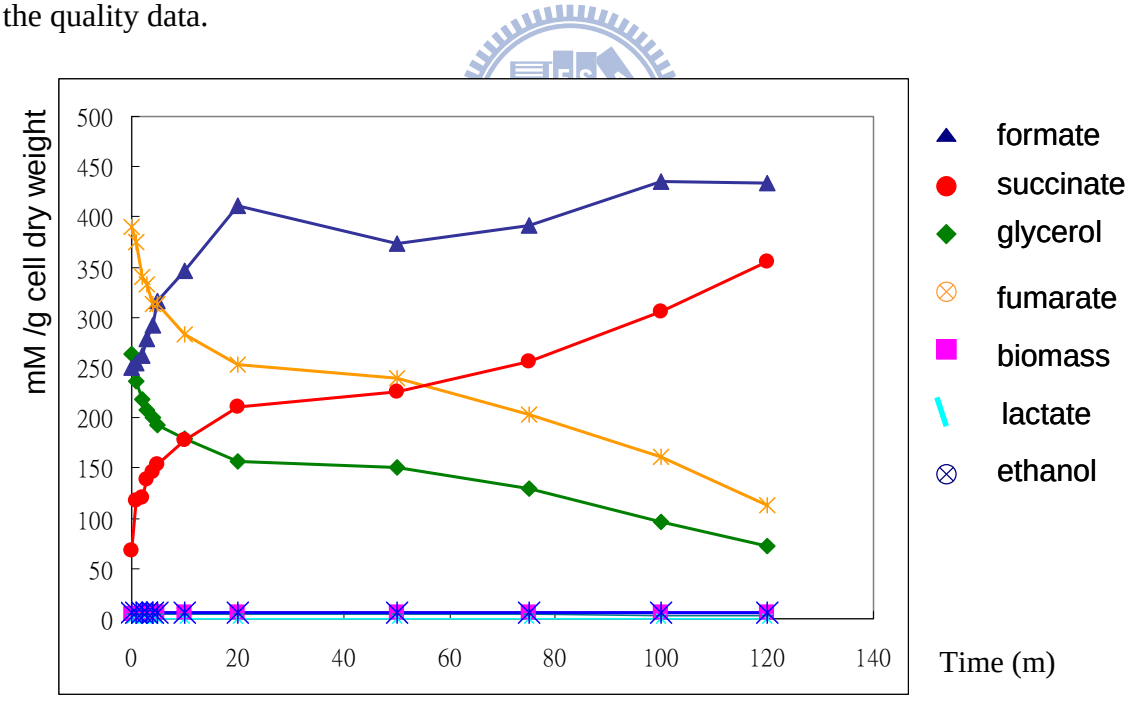


Figure 3.3 Anaerobic utizilation of glycerol by E. coli supplemented with fumarate

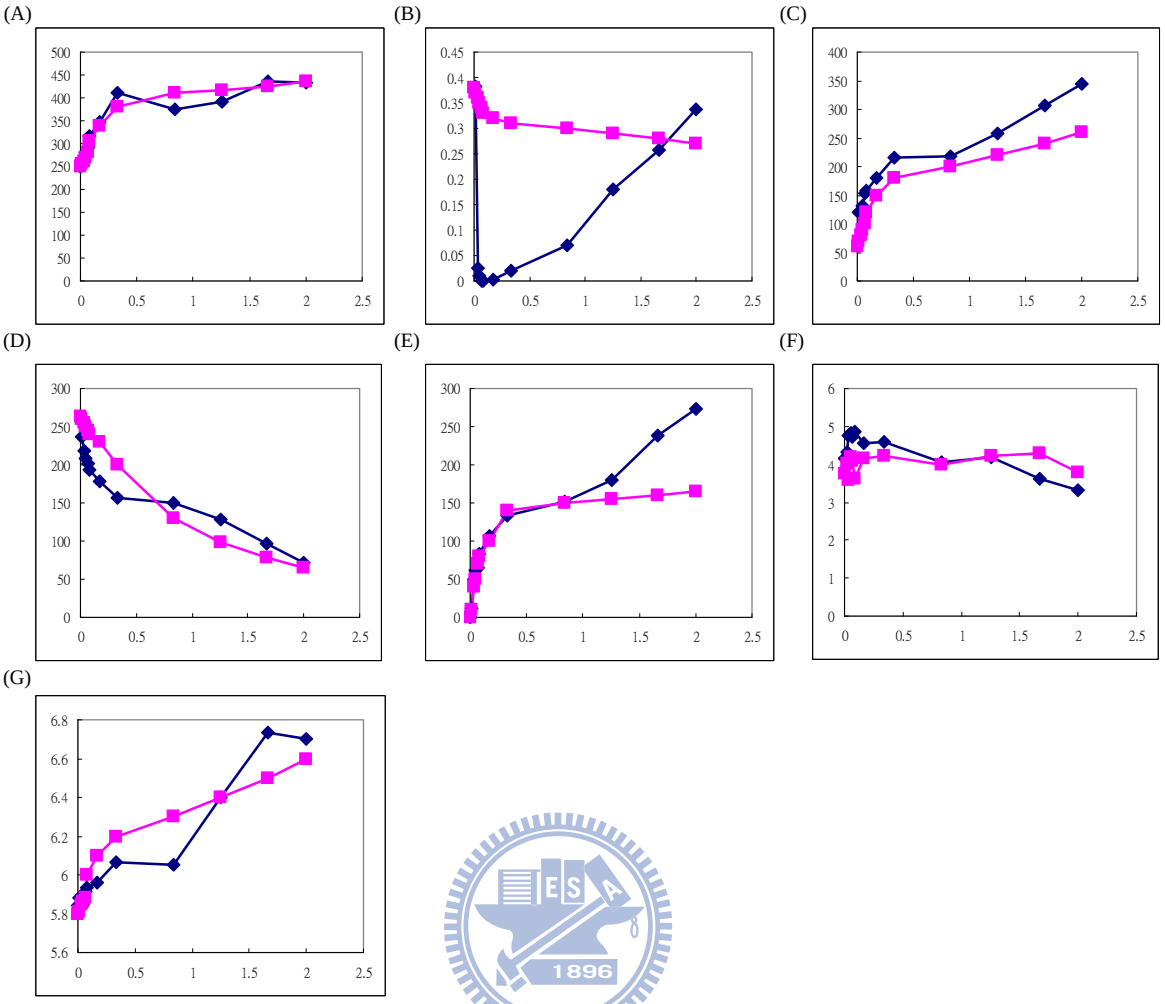


Figure 3.4 Contrast of experimental data in anaerobic utilization of glycerol by E. coli supplemented with fumarate (dark blue diamond) and simulation data (pick square) (A) formate (B) lactate (C)succinate(D)glycerol(E)fumarate consume(F)acetate(G)ethanol

In Figure 3.3, formate and succinate are major end product, and the concentrations of glycerol decrease parallel with fumarate. Moreover, fumarate respiration net reactions contain H_2 + Fumarate --, Succinate and HCO_2^- + Fumarate + H + ~ CO2 + Succinate, that two reactions end product agreement with experimental data [31].

The simulation shown in Figure 3.4, the direct comparisons experimental data perturbation more than simulation because of the simulation prefer the linear values than large variations values.

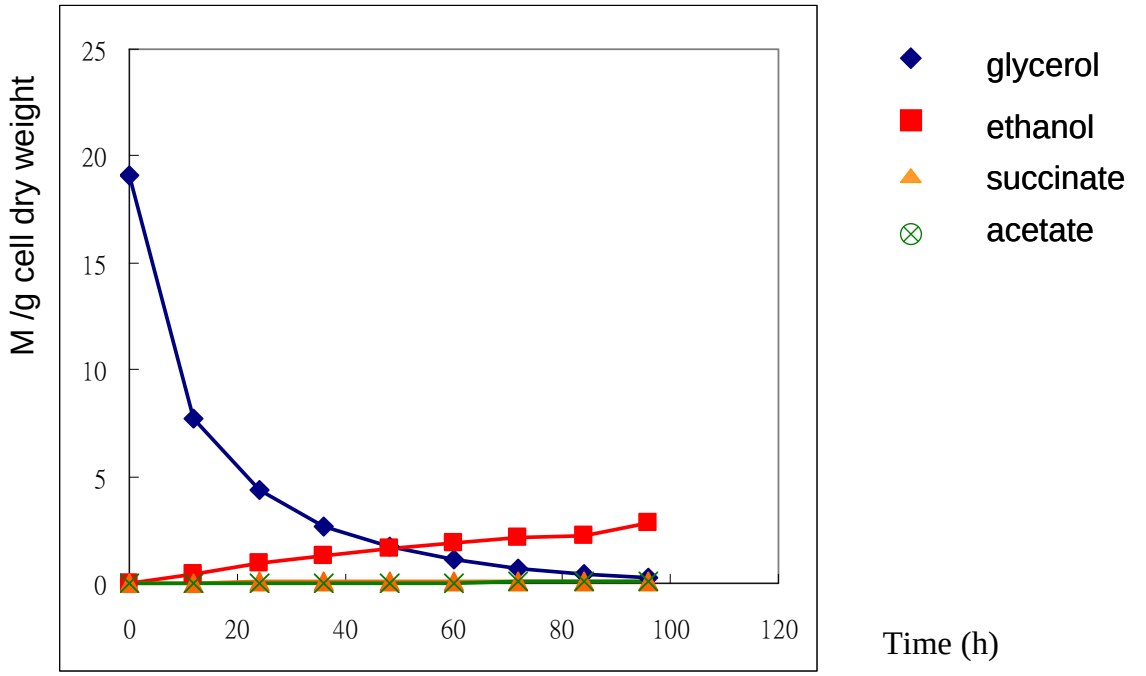


Figure 3.5 Fermentation of glycerol by E. coli supplemented with tryptone

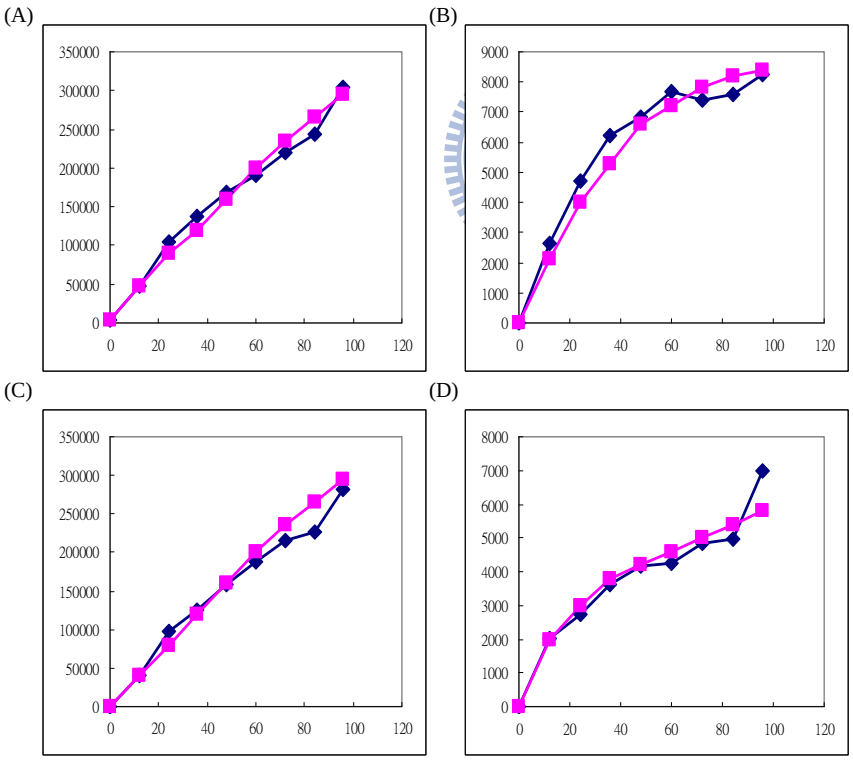


Figure 3.6 Contrast of experimental data in fermentation of glycerol by E. coli supplemented with tryptone (dark blue diamond) and simulation data (pick square) (A) glycerol consume (B) ethanol (C) succinate (D) acetate.

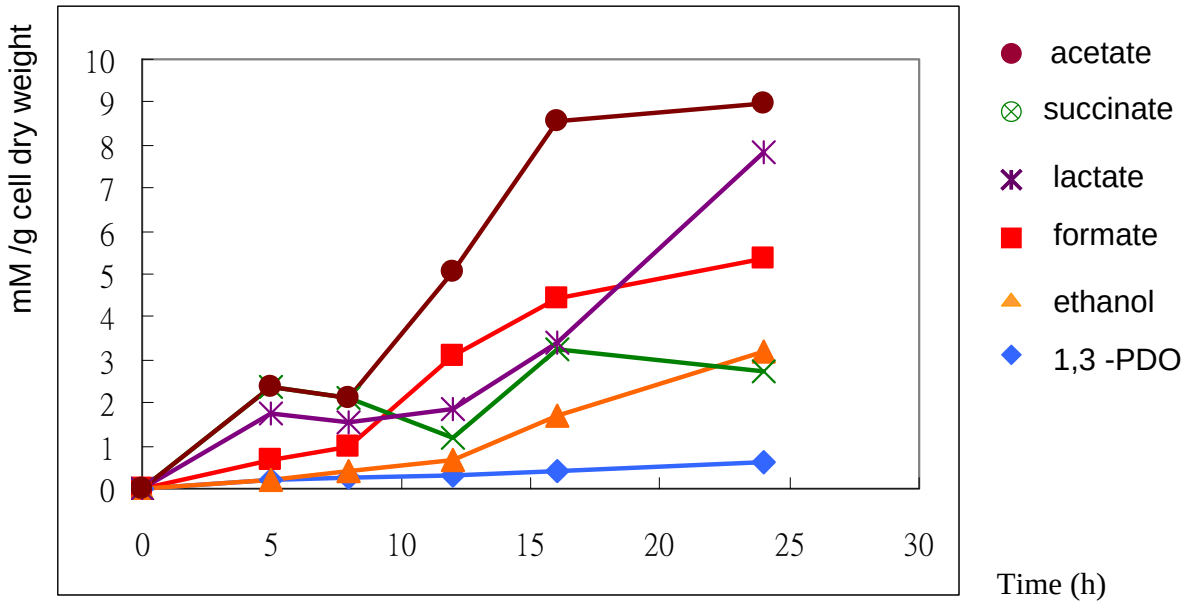


Figure 3.7 Fermentation of glycerol by E. coli expressed foreign gene dhaB from *Klebsiella pneumoniae.*

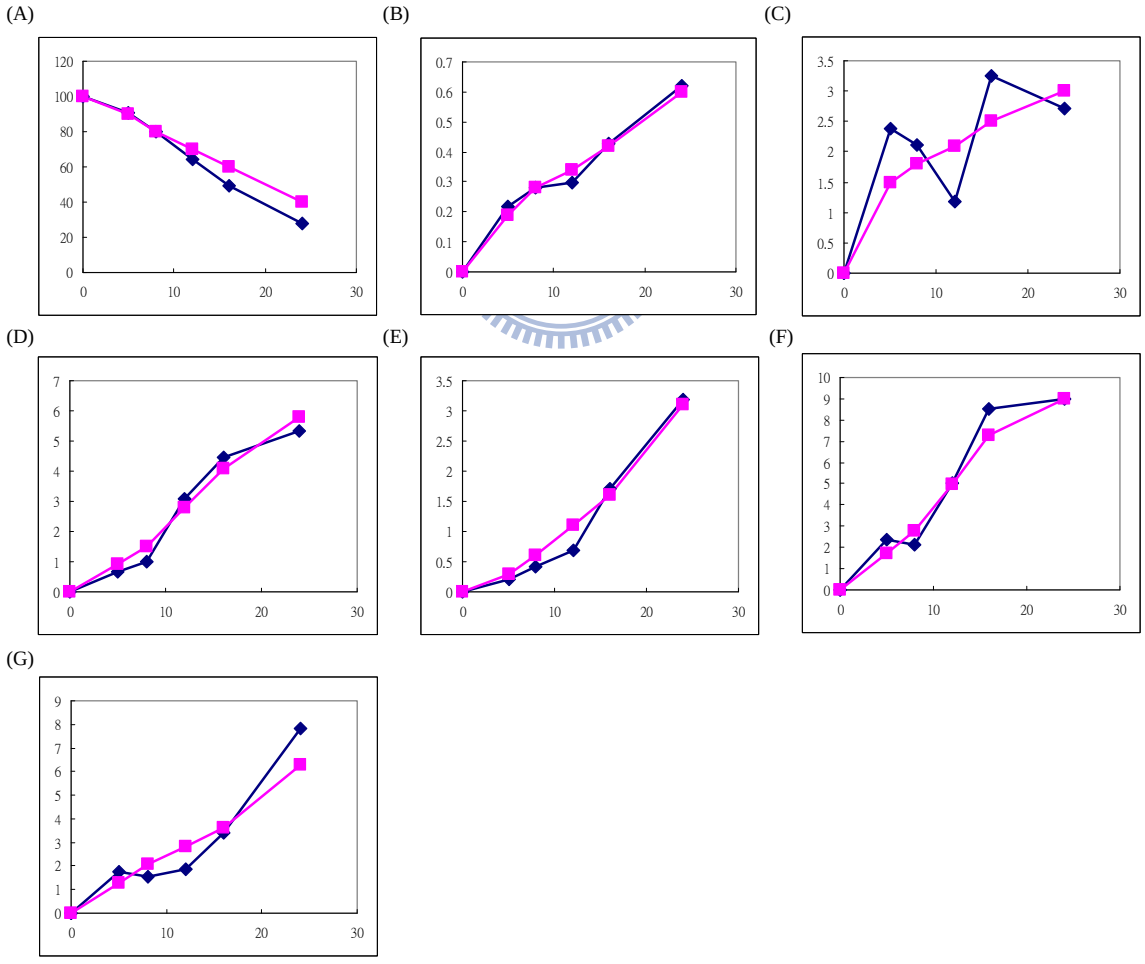


Figure 3.8 Contrast of experimental data in fermentation of glycerol by E. coli expressed foreign gene dhaB from *Klebsiella pneumoniae* (dark blue diamond) and simulation data (pick square) (A) glyceorl (B) 1,3-PDO (C) succinate (D) formate (E) ethanol (F) acetate (G) lactate.

3.6 Summery

This section extends above part of elementary flux mode, and we construct the dynamic model. This model contains the previous study method called hybrid method combine the elementary flux mode and enzyme kinetic base equation. Let the model work, we have to identify parameters in kinetic equation. Therefore the experimental data for glycerol anaerobic utilization in different condition was used to test the parameters can successfully push the model to simulate data that similar to experimental data.

The results of simulation are very similar to experimental data, the model we construct are valuable model to predict metabolic network in glycerol anaerobic utilization.

Chapter 4 Model verification and validation

4.1 Introduction

4.1.1 Parameter sensitivity analysis

The study about how to assign the uncertainty in the output of a mathematical model to different sources of variation in the input of a mathematical model either qualitatively or quantitatively is called sensitivity analysis (SA).[32]

Generally, when studies include some form of mathematical modeling, uncertainty and sensitivity analyses was usually used to check the robustness of a study. Uncertainty analysis studies the overall uncertainty in the conclusions of the studies, while sensitivity analysis identifies which source of uncertainty weights more in the conclusions. Several guidelines for impact assessment or for modeling have used sensitivity analysis as a tool to make sure the reliability of the modeling or assessment. [32]

4.1.2 Correlation of flux and parameters

The correlation between two homogenous or heterogenous data indicated the perturbation of one part, the effect affect to others. This information can tell us the intensity of factory we concern, for example, hair style and gender are related; long hair people tend to be girl than short hair people. But the relation is not 100 percentages.

Correlation applies for quantifiable data that numbers contain significant, usually quantities of some sort.

4.2 Results

In this section, we demonstrate the verification of model's parameters and provide one case study to promote creditability of glycerol fermentation model. It can be seen that each parameters how much of strength influence the metabolites concentration. Because of values disproportion, parameters distributions incline to distinguish into confusion part and limitation part. More specifically, the similarity of EFMs affect the strength is worth while to discussion.

4.2.1 Parameters analysis and correlation coefficient between EFMs

The parameters have a primary role in mathematical model and greatly influence how the cell behavior be simulated.

About the control effect of parameters, Figure 4.1 illustrate the strange of parameters can be separated to a subset that has high coefficient, although the parameters didn't have high coefficient for every metabolite, tend to prefer high coefficient. Because of the trend, select one of mode that correspond high coefficient parameter's shown in Figure 4.4.Compare with Figure 2.11 and Figure 2.12, the reactions are a lot of different such as produce acetate and succinate ,and no ethanol produce. Studies should be undertaken to determine the frequency of each reactions operating shown in Table 4.1.the high frequency reactions such as F16BP = F6P, GL6P = 6PG, 6PG + NADP = R5P + CO2 + NADPH, and glycolysis pathway are more consistent than the fermentative pathway , that explain the major trunk pathway and branch pathway in high influence EFMs.

Table 4.1 Reaction frequency of EFMs for glycerol fermentation plus tryptone: 33~133

GG1	GG3	GG4	GG11	GG12	GG13	PPP2	PPP3	TCA1	TCA2r	FR1
0	0	1	0.87	0	0.48	1	1	0.95	0.95	0.58
FR2	FR3	ANA1	ANA2	ANA3	FEM1	FEM2	FEM7	FEM8	FEM3	FEM5
0.63	0.63	1	0.19	0	0.49	0.19	0.25	0.25	0.13	0.12
FEM6	FEM4	FEM9	EDP1	EDP2	XYL1	XYL2	GAL1	MAN1	MAN2	ARA1
0.25	0.49	0.13	0	0	0	0	0	0	0	0
BIO	OPM4r	FC2	TRA1	TRA2	TRA3	TRA4	TRA5	TRA6	TRA7	GLB1
0.95	0.51	0	0.25	0.44	0.95	0.13	0.69	0	0.55	0.24
GLB2	TRA8	TRA9	TRA10	GLYD1	GLYD2	GLYD3	GLYD4	OPM3	TRYP	GG2r
0.24	0	0	0	1	0	1	1	0	1	1
GG5r	GG6r	GG7r	GG8r	GG9r	GG10r	PPP1	PPP4r	PPP5r	PPP6r	PPP7r
1	1	1	1	1	1	1	0.69	1	0.68	0.68
PPP8r	TCA3r	TCA4	FC1r							
0.68	0.95	0.95	1							

Moreover, the reaction rate is equal to parameters multiply elementary flux mode, the parameter value is an important characteristic of reaction rate. Figure 4.2 depict the parameters value express in logarithm and normal bar chart. It alteration of parameters in low coefficient part is small compare with high coefficient part in logarithm bar chart, and the average value of parameters in low coefficient part is larger than high coefficient part.

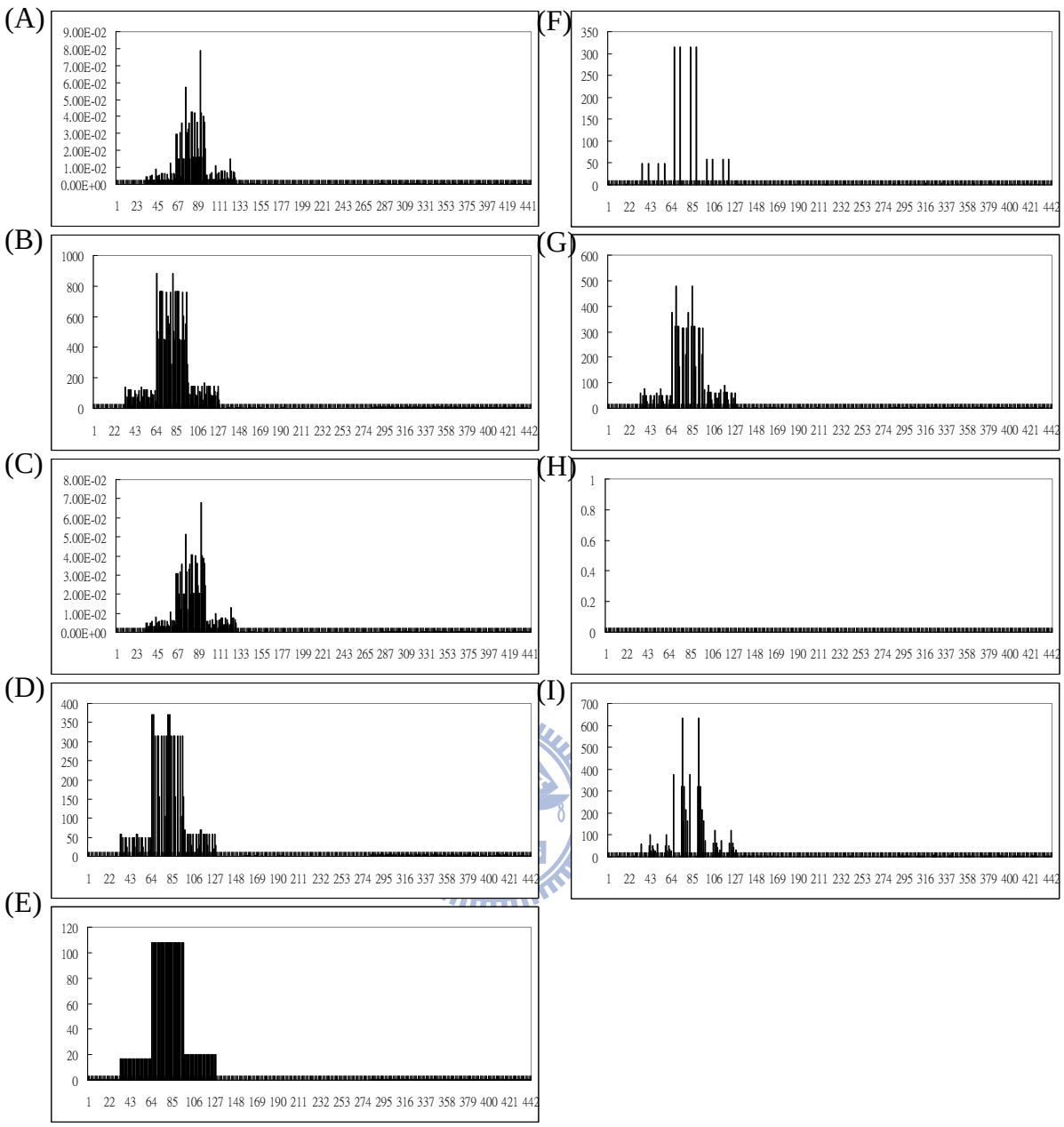


Figure 4.1 Coefficient of parameters and metabolites concentration: glycerol fermentation plus tryptone (A) tryptone (B) Ethanol (C) Succinate (D) Glycerol (E) NH3 (F) Formate (G) Biomass (H) Lactate (I) CO2.

Furthermore, we investigate the correlation coefficient of each EFMs, that can realize that every EFMs are similar or not. Figure 4.3 shows the correlation coefficient in glycerol fermentation plus tryptone, the red point represent high correlation coefficient, yellow point represent low correlation coefficient, and green represent negative correlation coefficient. There are many red square that reveal this part of EFMs is similar shown in Figure 4.3, and we collect three parts of EFMs that have high correlation coefficient.

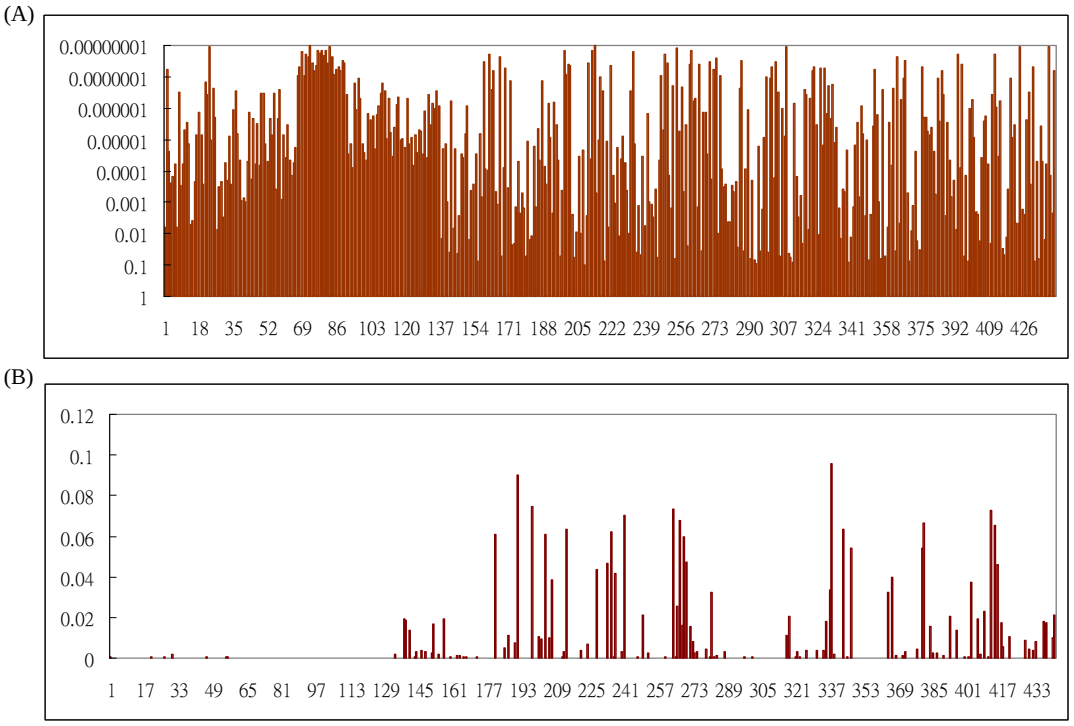


Figure 4.2 Parameters distribution : glycerol fermentation plus tryptone .

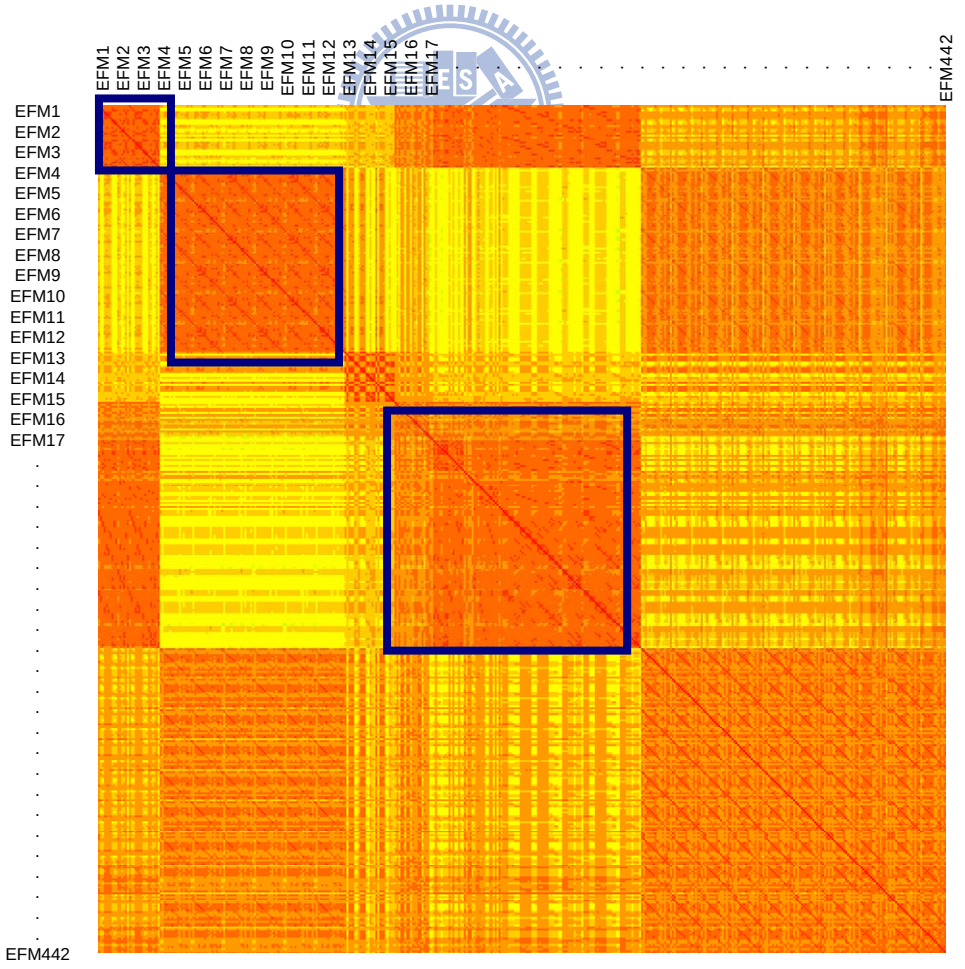


Figure 4.3 EFMs Correlation coefficient: glycerol fermentation plus tryptone.

Each of the three parts EFMs that we select one to compare with early work shown in Figure 4.5, Figure 4.6, and Figure 4.7. In Figure 4.5, the major product is acetate. In Figure 4.6, the major products are ethanol, biomass, and succinate. In Figure 4.7, it have the most less reaction that only convert glycerol to succinate and ethanol ,but this EFMs didn't growing biomass. Above all, the cluster of EFMs matrix provides a possible thinking that performs the a few EFMs to stand for whole systems EFMs.

The frequency of high correlation coefficient part shown in Table 4.2-4.4, focus on mode 151 – mode 282, there is none of the reaction about biomass growing, and the less flow in pentose phosphate pathway and ratio of ethanol in fermentative produce is increased. That cluster of EFMs stand for high yields EFMs lead us to further research on the question of how to balance the cell growing and increase the ethanol yields.

Table 4.2 Reaction frequency of EFMs for glycerol fermentation plus tryptone: 1~32

GG1	GG3	GG4	GG11	GG12	GG13	PPP2	PPP3	TCA1	TCA2r	FR1
0	0	1	0.87	0	0.47	0	0	1	1	0.57
FR2	FR3	ANA1	ANA2	ANA3	FEM1	FEM2	FEM7	FEM8	FEM3	FEM5
0.63	0.63	1	0.2	0	0.53	0.17	0.23	0.23	0.13	0.13
FEM6	FEM4	FEM9	EDP1	EDP2	XYL1	XYL2	GAL1	MAN1	MAN2	ARA1
0.27	0.53	0.13	0	0	0	0	0	0	0	0
BIO	OPM4r	FC2	TRA1	TRA2	TRA3	TRA4	TRA5	TRA6	TRA7	GLB1
1	0.53	0	0.27	0.4	1	0.13	0.7	0	0.53	0.27
GLB2	TRA8	TRA9	TRA10	GLYD1	GLYD2	GLYD3	GLYD4	OPM3	TRYP	GG2r
0.27	0	0	0	1	0	1	1	0	1	1
GG5r	GG6r	GG7r	GG8r	GG9r	GG10r	PPP1	PPP4r	PPP5r	PPP6r	PPP7r
1	1	1	1	1	1	0	1	1	1	1
PPP8r	TCA3r	TCA4	FC1r							
1	1	1	1							

Table 4.3 Reaction frequency of EFMs for glycerol fermentation plus tryptone: 33~133

GG1	GG3	GG4	GG11	GG12	GG13	PPP2	PPP3	TCA1	TCA2r	FR1
0	0	1	0.87	0	0.48	1	1	0.95	0.95	0.58
FR2	FR3	ANA1	ANA2	ANA3	FEM1	FEM2	FEM7	FEM8	FEM3	FEM5
0.63	0.63	1	0.19	0	0.49	0.19	0.25	0.25	0.13	0.12
FEM6	FEM4	FEM9	EDP1	EDP2	XYL1	XYL2	GAL1	MAN1	MAN2	ARA1
0.25	0.49	0.13	0	0	0	0	0	0	0	0
BIO	OPM4r	FC2	TRA1	TRA2	TRA3	TRA4	TRA5	TRA6	TRA7	GLB1
0.95	0.51	0	0.25	0.44	0.95	0.13	0.69	0	0.55	0.24
GLB2	TRA8	TRA9	TRA10	GLYD1	GLYD2	GLYD3	GLYD4	OPM3	TRYP	GG2r
0.24	0	0	0	1	0	1	1	0	1	1
GG5r	GG6r	GG7r	GG8r	GG9r	GG10r	PPP1	PPP4r	PPP5r	PPP6r	PPP7r
1	1	1	1	1	1	1	0.69	1	0.68	0.68
PPP8r	TCA3r	TCA4	FC1r							
0.68	0.95	0.95	1							

Table 4.4 Reaction frequency of EFMs for glycerol fermentation plus tryptone: 151~282

GG1	GG3	GG4	GG11	GG12	GG13	PPP2	PPP3	TCA1	TCA2r	FR1
0	0.19	0.34	0.76	0.19	0.4	0.15	0	0.35	0.35	0.53
FR2	FR3	ANA1	ANA2	ANA3	FEM1	FEM2	FEM7	FEM8	FEM3	FEM5
0.57	0.57	0.81	0.3	0.19	0.41	0.13	0.28	0.28	0.05	0.19
FEM6	FEM4	FEM9	EDP1	EDP2	XYL1	XYL2	GAL1	MAN1	MAN2	ARA1
0.28	0.41	0.09	0.15	0.15	0	0	0	0	0	0
BIO	OPM4r	FC2	TRA1	TRA2	TRA3	TRA4	TRA5	TRA6	TRA7	GLB1
0	0.45	0.19	0.28	0.4	0	0.05	0.67	0	0.54	0.35
GLB2	TRA8	TRA9	TRA10	GLYD1	GLYD2	GLYD3	GLYD4	OPM3	TRYP	GG2r
0.35	0	0	0	1	0	1	1	0.19	0.91	0.15
GG5r	GG6r	GG7r	GG8r	GG9r	GG10r	PPP1	PPP4r	PPP5r	PPP6r	PPP7r
0.15	0.98	0.98	0.98	0.98	0.98	0.15	0	0	0	0
PPP8r	TCA3r	TCA4	FC1r							
0	0.35	0	0.15							

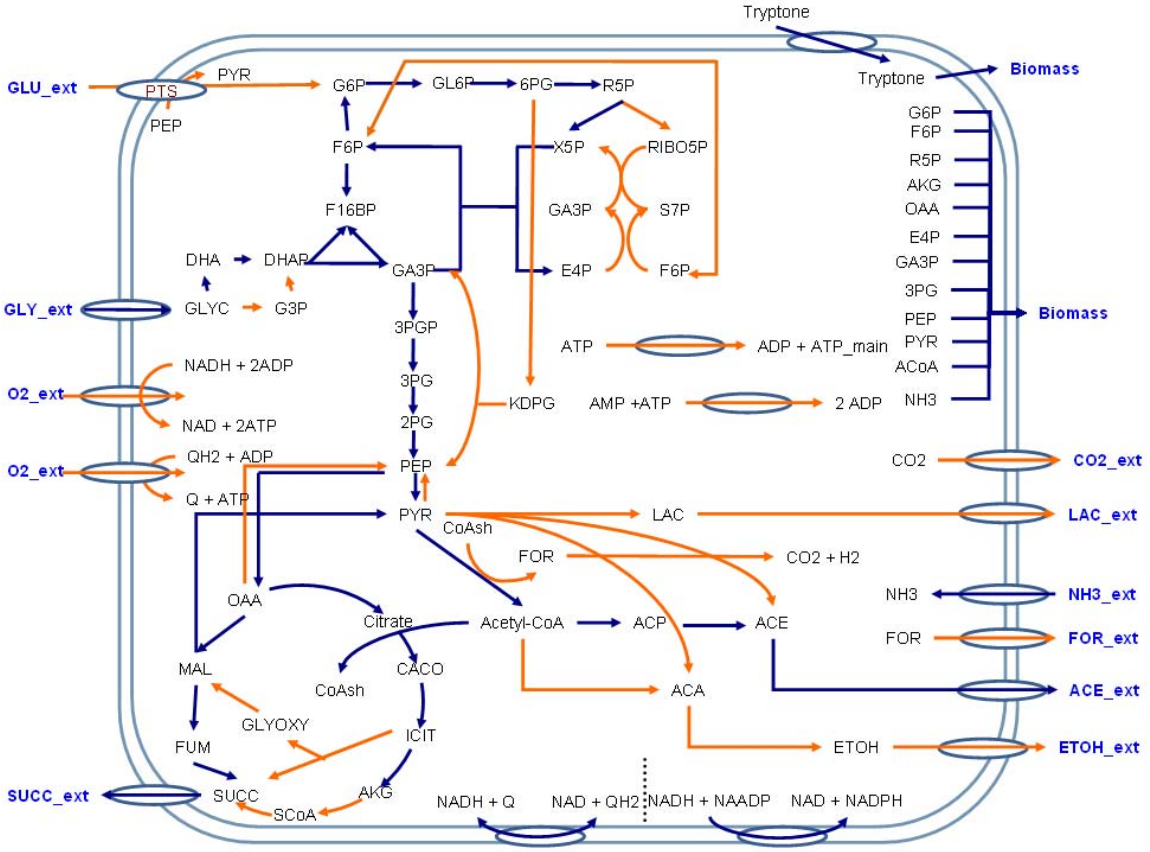


Figure 4.4 The mode No.81 in glycerol fermentation in medium plus tryptone.

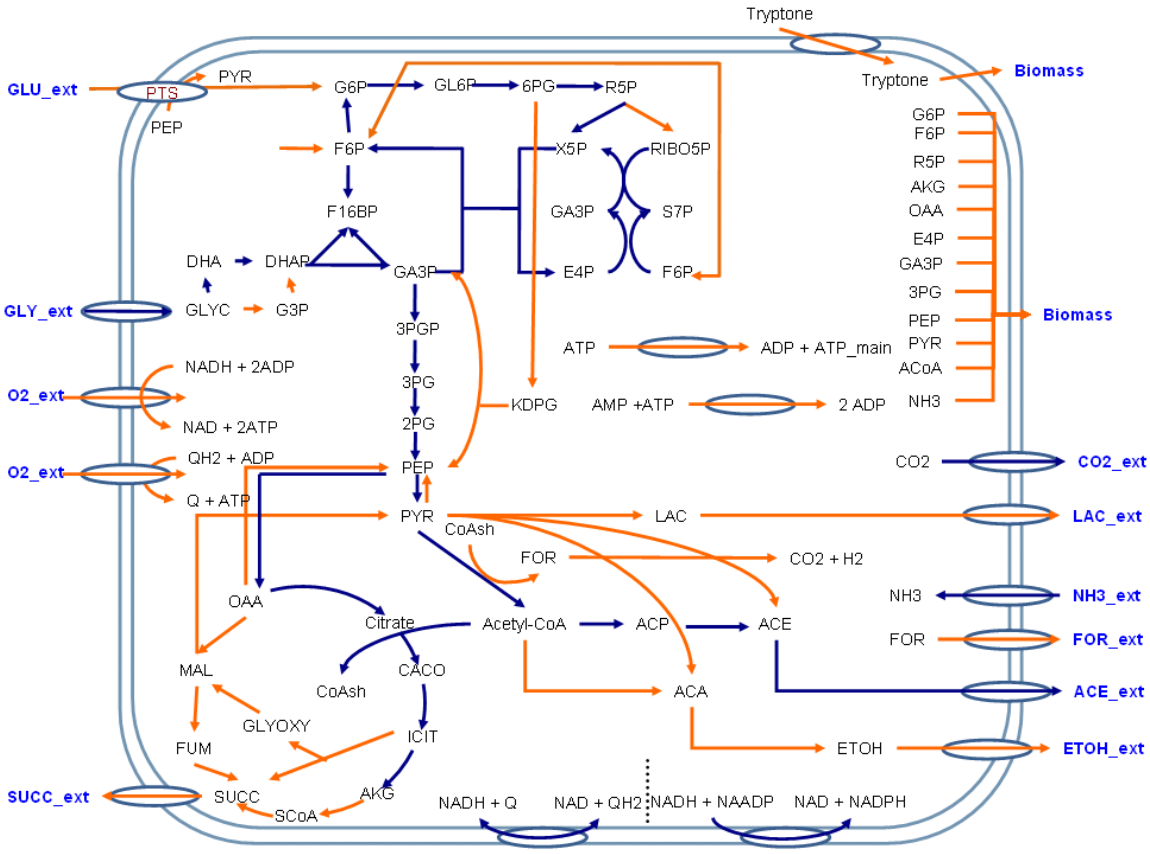


Figure 4.5 The mode No.18 in glycerol fermentation in medium plus tryptone.

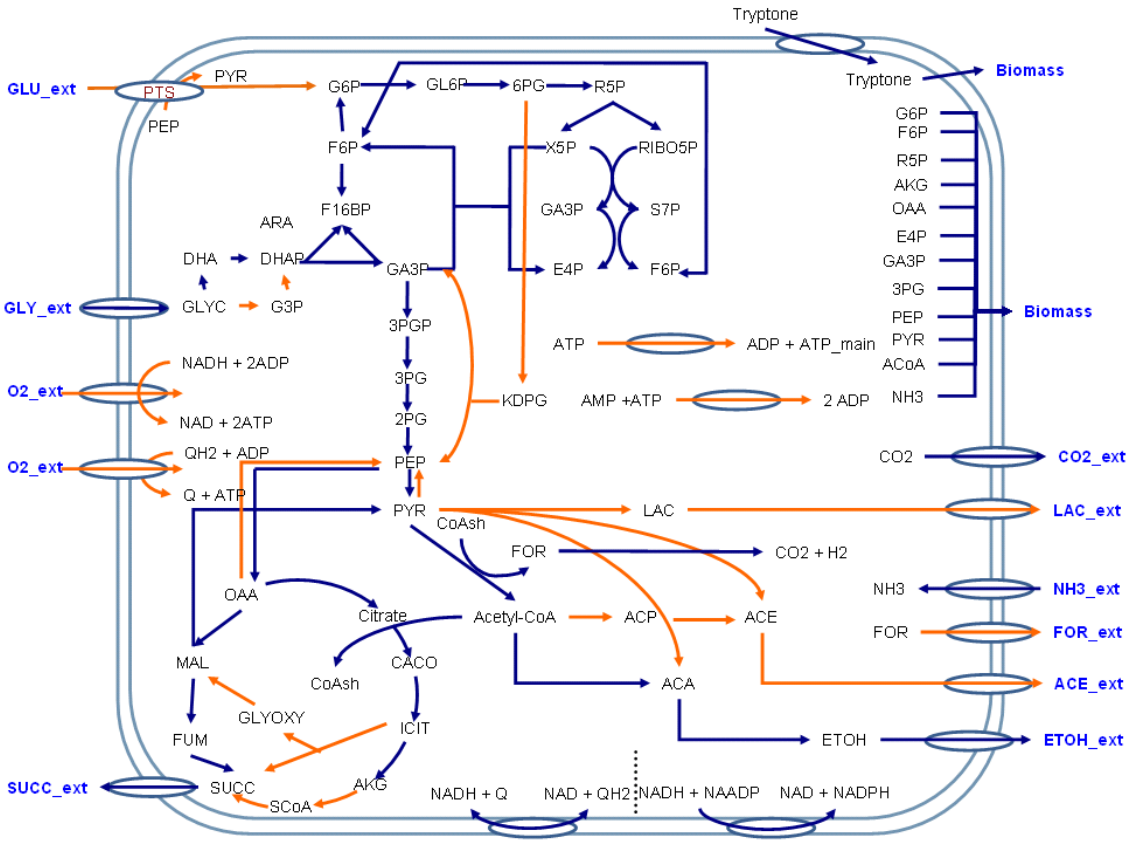


Figure 4.6 The mode No.100 in glycerol fermentation in medium plus tryptone.

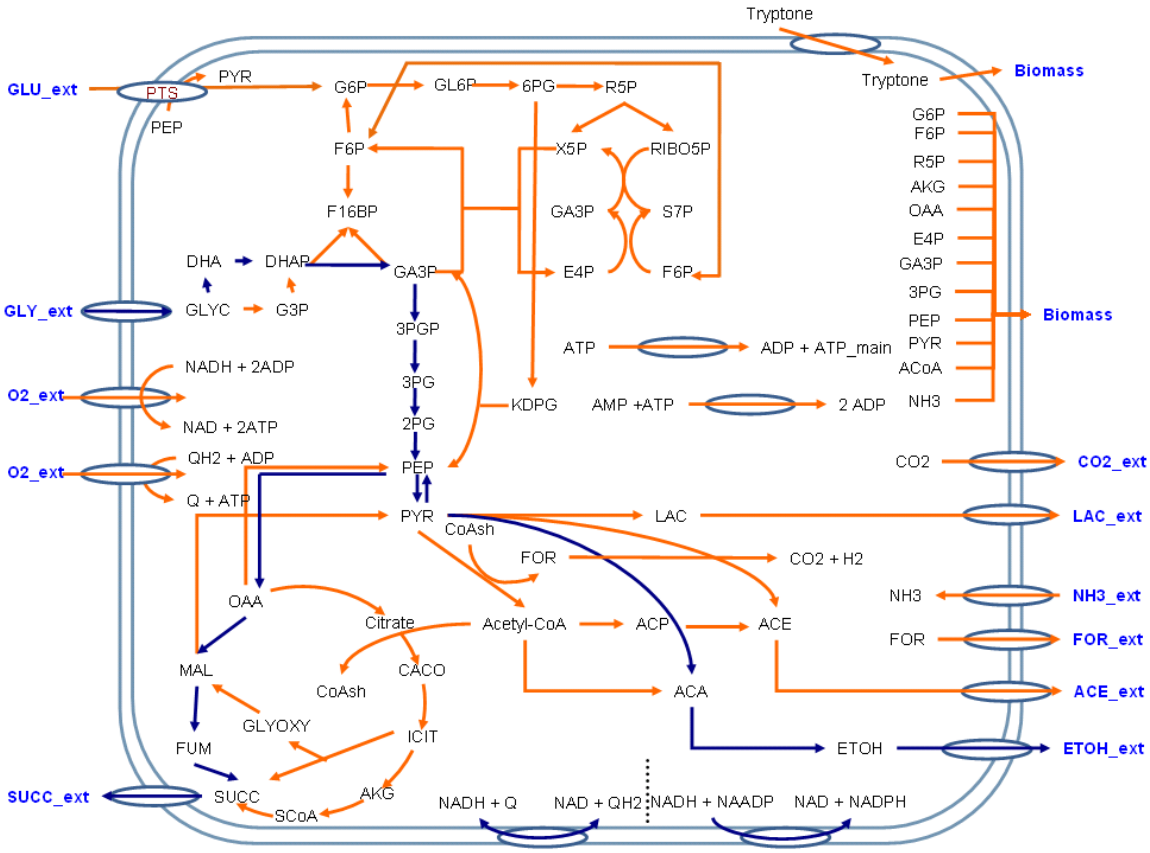


Figure 4.7 The mode No.220 in glycerol fermentation in medium plus tryptone.

In other condition, glycerol fermentation plus furmarate, there are also high coefficient subset and low high coefficient subset parameters with metabolites concentration.. Although the high coefficient parameters modes influence averagely, it have less effect to the exception metabolite. Using these variations, the extreme condition and phenomenon can be revealed. So the cluster of similar EFMs can not use few amounts of EFMs to represent the whole systems.

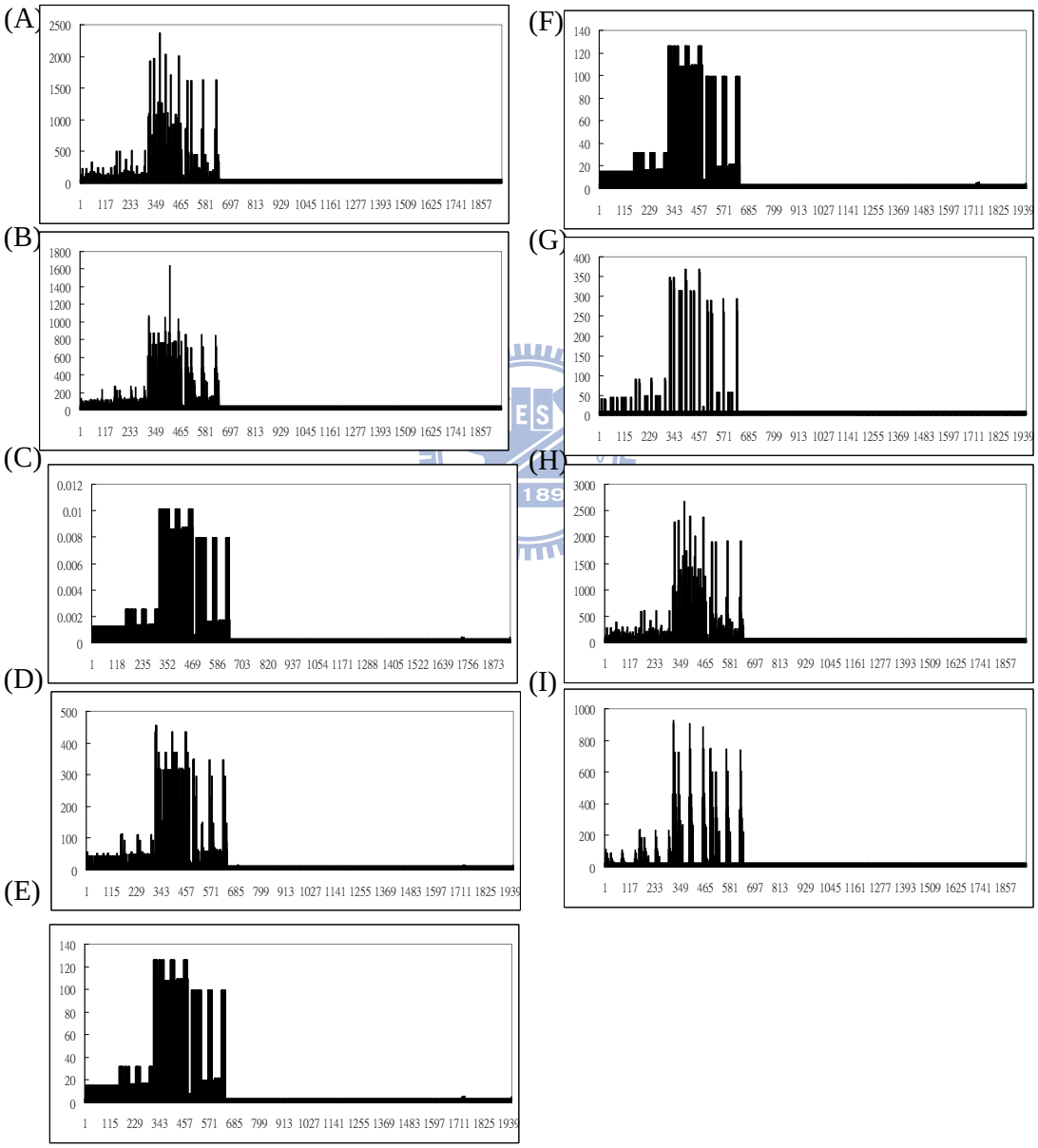


Figure 4.8 Coefficient of parameters and metabolites concentration: glycerol fermentation plus furmarate (A) Furmarate (B) Ethanol (C) Succinate (D) Glycerol (E) NH3 (F) Formate (G) Biomass (H) Lactate (I) CO2.

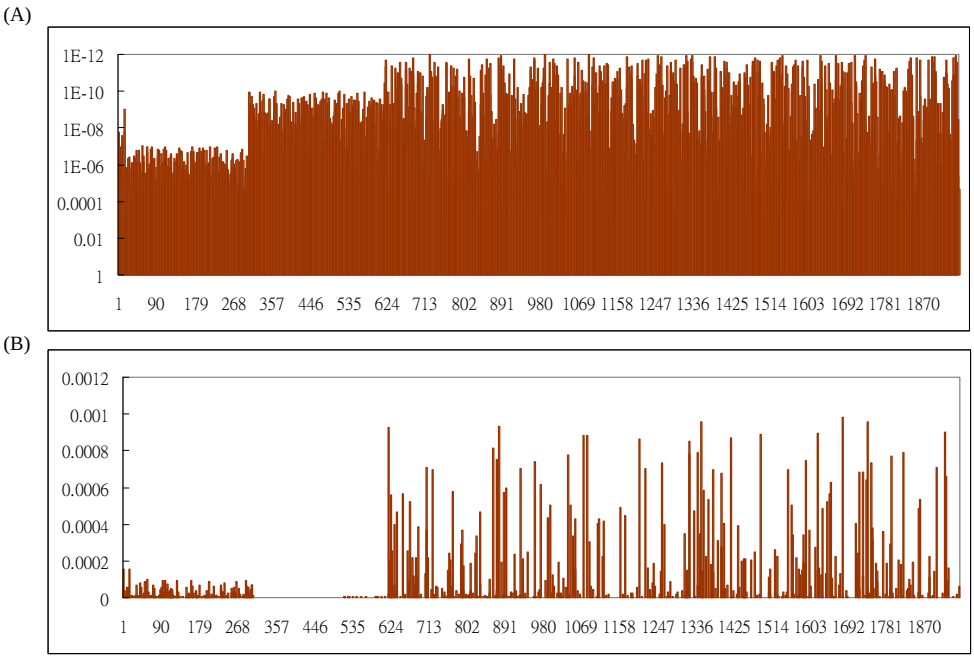


Figure 4.9 Parameters distribution: glycerol anaerobic utilization plus fumarate

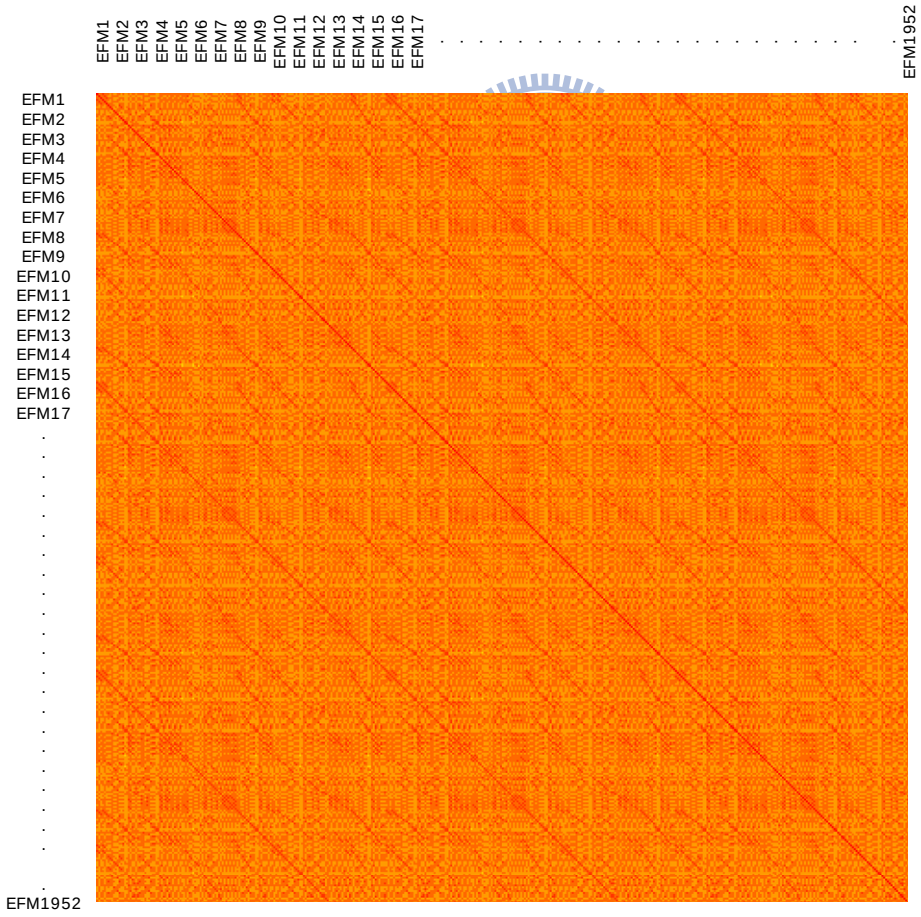


Figure 4.10 EFMs Correlation coefficient: glycerol anaerobic utilization plus fumarate

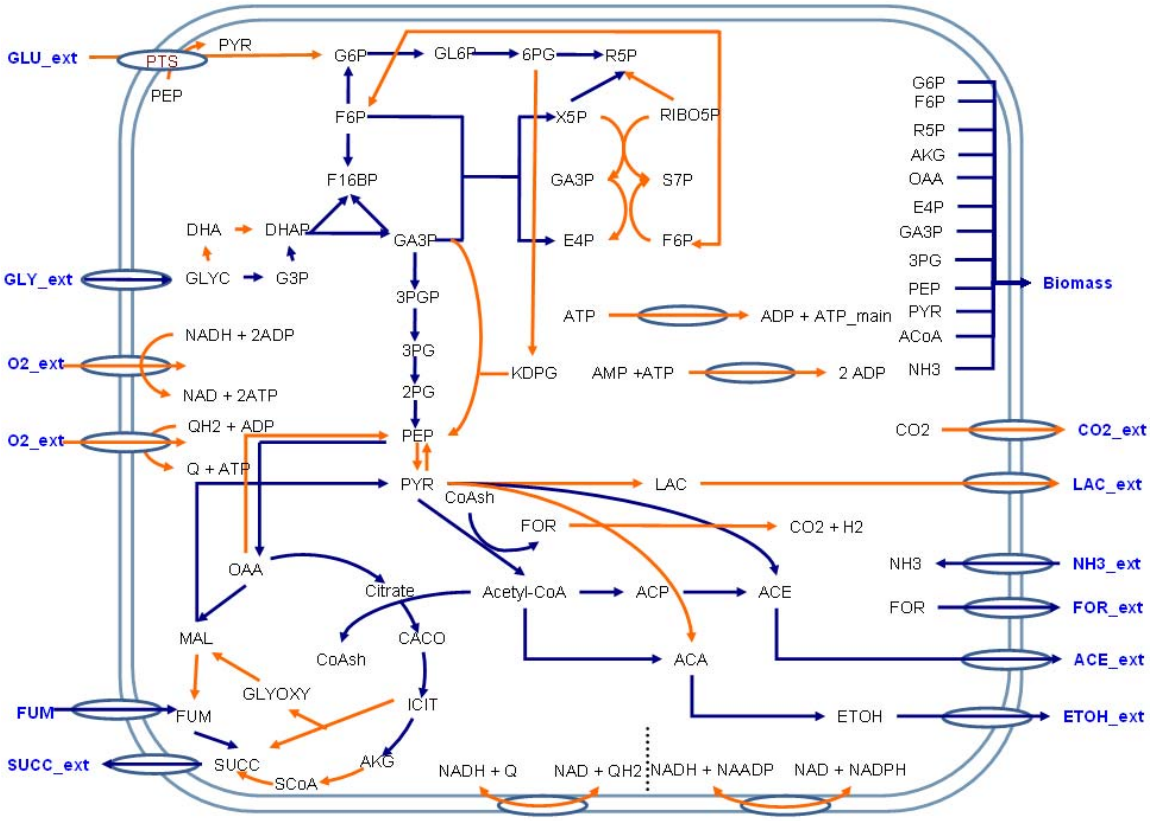


Figure 4.11 The mode No.320 in glycerol fermentation in medium plus fumarate

The EFMs correlation coefficient about glycerol anaerobic utilization plus fumarate shown in Figure 4.10, that correlation coefficient seems very unanimous blot. The possible reason about EFMs of glycerol fermentation in medium plus fumarate similarity is that fumarate make the reactions of metabolic pathway similar in fumarate related part such as malate convert to fumarate , fumarate convert to succinate , and all of EFMs have succinate as end product.

Finally, analysis of *E.coli* ferment glycerol that expressed foreign gene dhaB from *Klebsiella pneumoniae.* In Figure 4.12, the value of coefficient in ethanol and 1,3-propanediol are higher than others, that explain the major product of recombinant *E.coli* are ethanol and 1,3-propanediol.becuase of the reactions produce ethanol and 1,3-propanediol that coproduce redox cofactor such as NADH or NAD+. The ratio of NADH and NAD+ are decisive role for cell organization, so intracellular balance about NADH and NAD+ were controlled very careful and attentive.

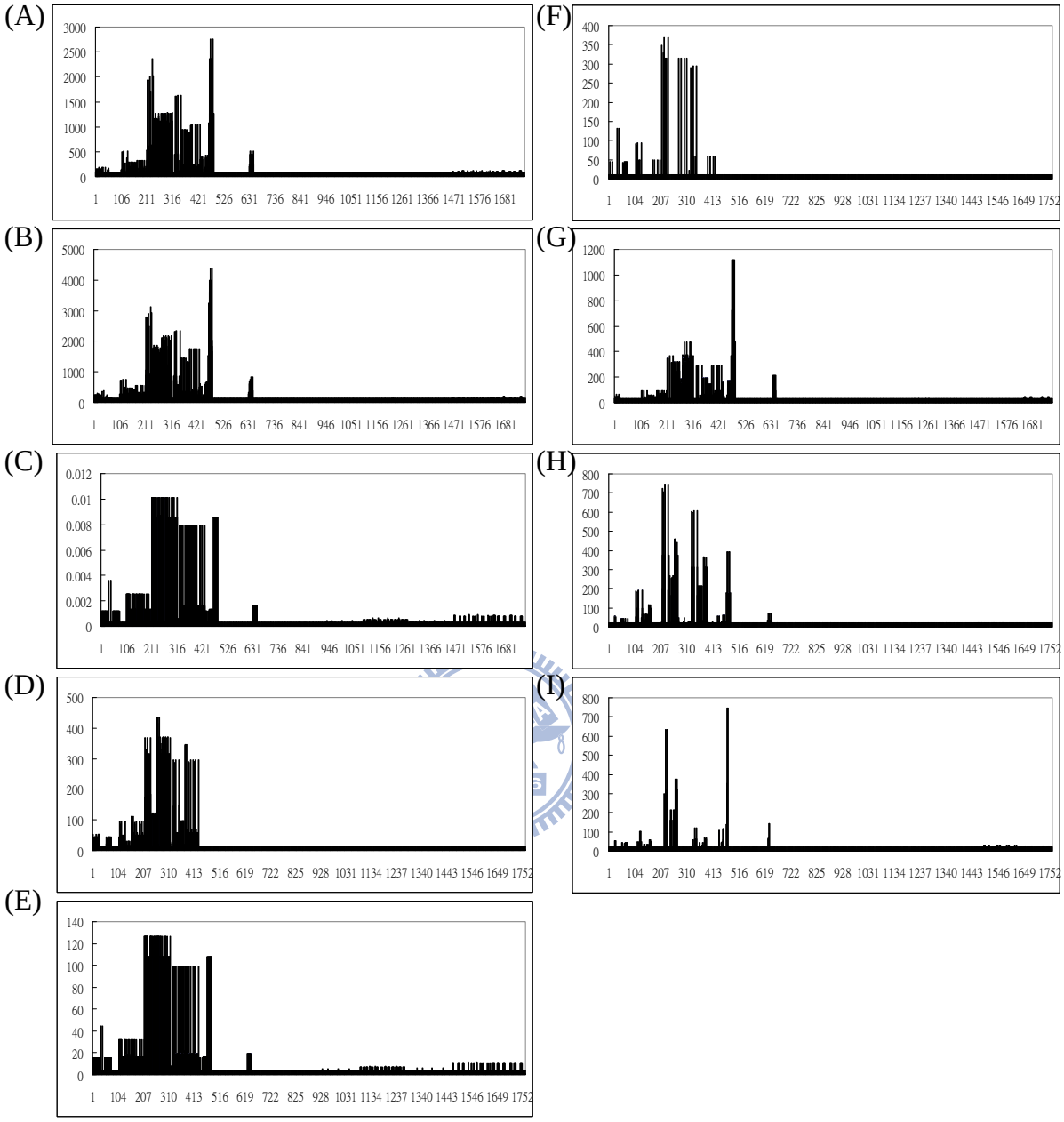


Figure 4.12 Coefficient of parameters and metabolites concentration: glycerol fermentation plus furmarate

(A) 1,3 - PDO (B) Ethanol (C) Succinate (D) Glycerol (E) NH3 (F) Formate (G) Biomass (H)Lactate(I)CO2

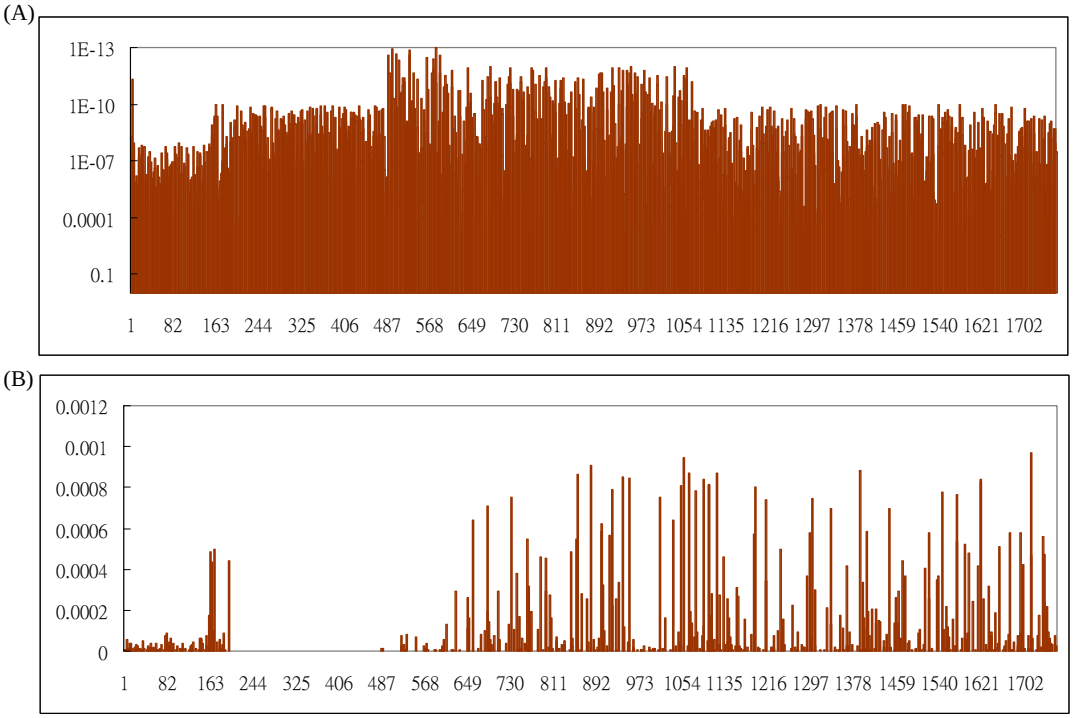


Figure 4.13 Parameters distribution: glycerol fermentation express foreign gene

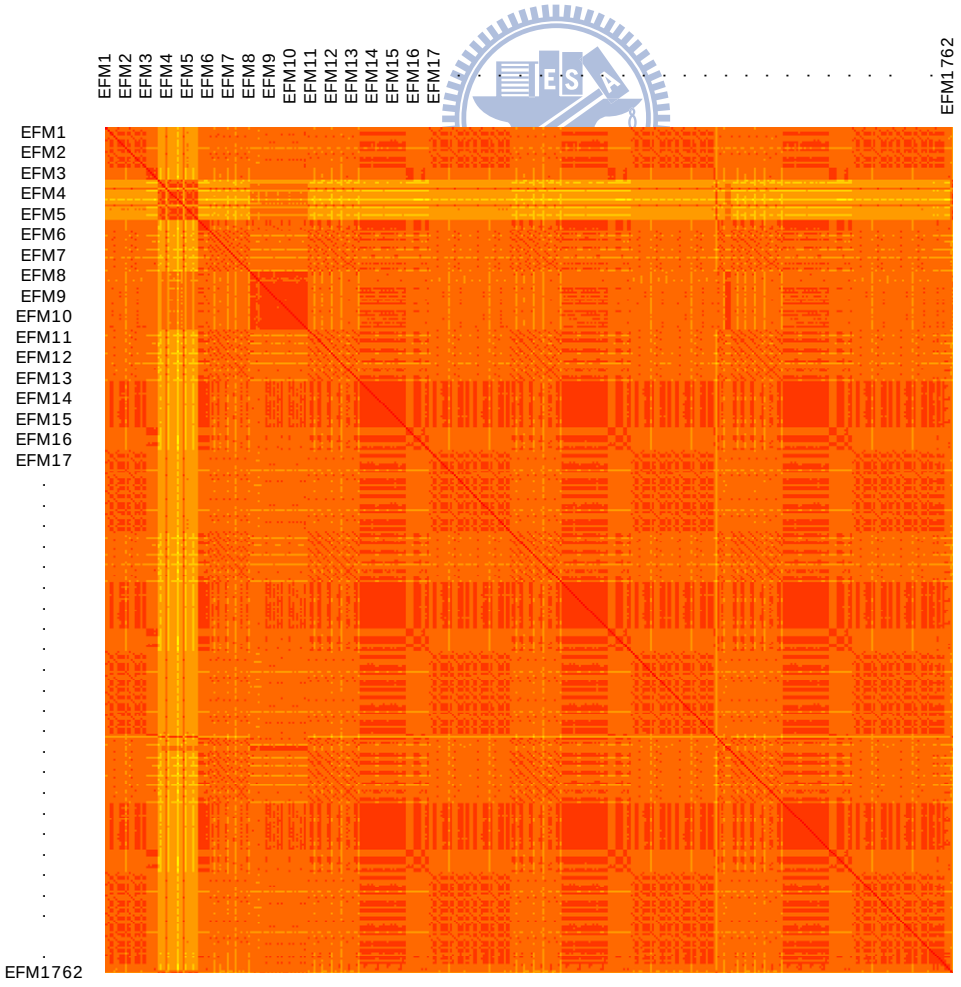


Figure 4.14 EFMs Correlation coefficient: glycerol fermentation express foreign gene.

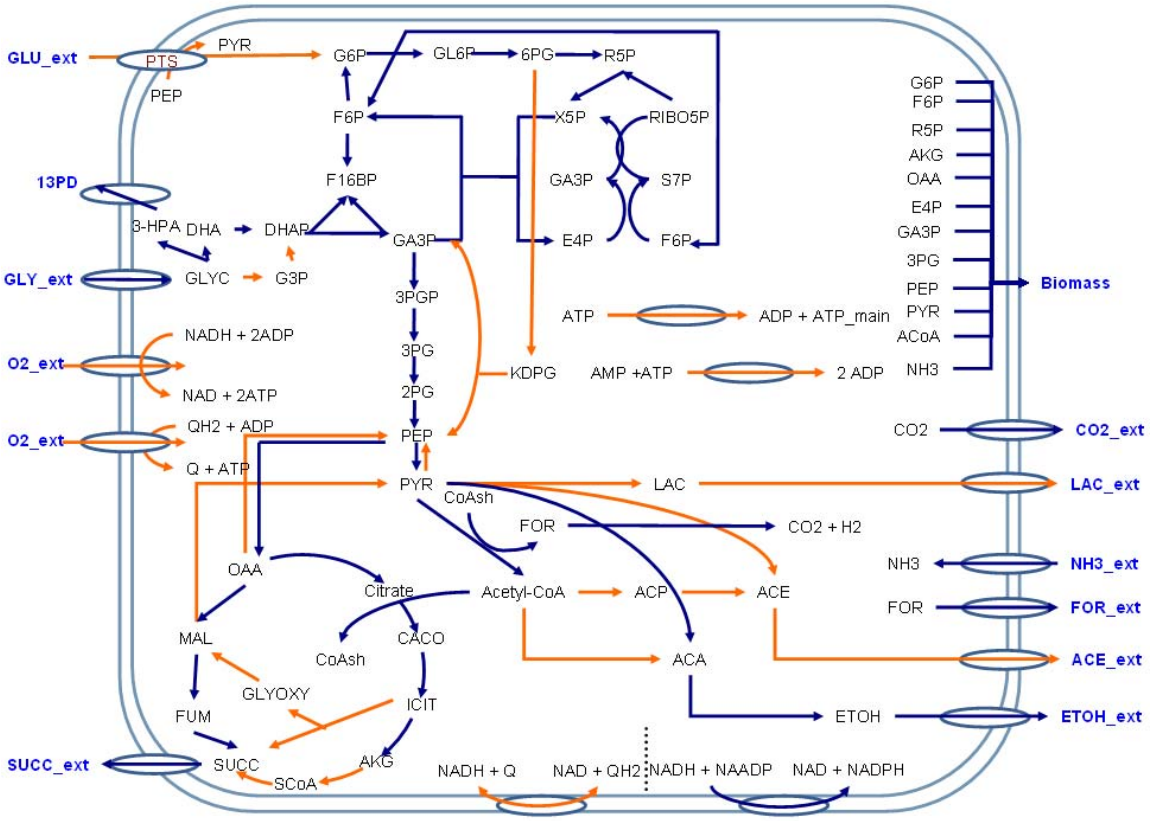


Figure 4.15 The mode No236 in glycerol fermentation expressed foreign gene dhaB.

4.2.2 Case study

The previous study's strategy for the metabolic engineering for glycerol fermentation in *E.coli* was changed the gene expression such as over expression or knout out, since the experiments for mortify the *E.coli* genes, raise the yield ethanol and glycerol uptake rate. They induced the GldA and DHAK gene that convert glycerol to dihydorxyacetone and dihydorxyacetone to dihydroxuacetone phosphate. Another varying are blocking FRD for convert fumarate to succinate, FHL for convert formate to CO2 and H2, and PTA for convert Acetyl-Coenzyme-A to acetyl-phosphate.[33]

We chose the case of FHL delete or not to verify our model quality of simulation, first we select the elementary flux mode that related to formate hydrogen lyase reactions, which contain 245 modes. When deleting a lot of modes send the all of metabolite decrease expect

formate, correspond to Figure 4.18 Left, also the whole systems reactions rate decrease, the converting ratio from glycerol to other metabolite alter a few, however, the whole systems include biomass growing rate decline .

Above part shown in Figure 4.1 (H), all elementary flux mode did not prefer formate generation, so their coefficient with formate concentration are very low.

We simulate the formate hydrogen lyase deletion shown in Figure 4.17 (right), although the rigorous values of biomass growing are different from experimental data, and the cell behaviors are entirely consistent with research in previous studies.

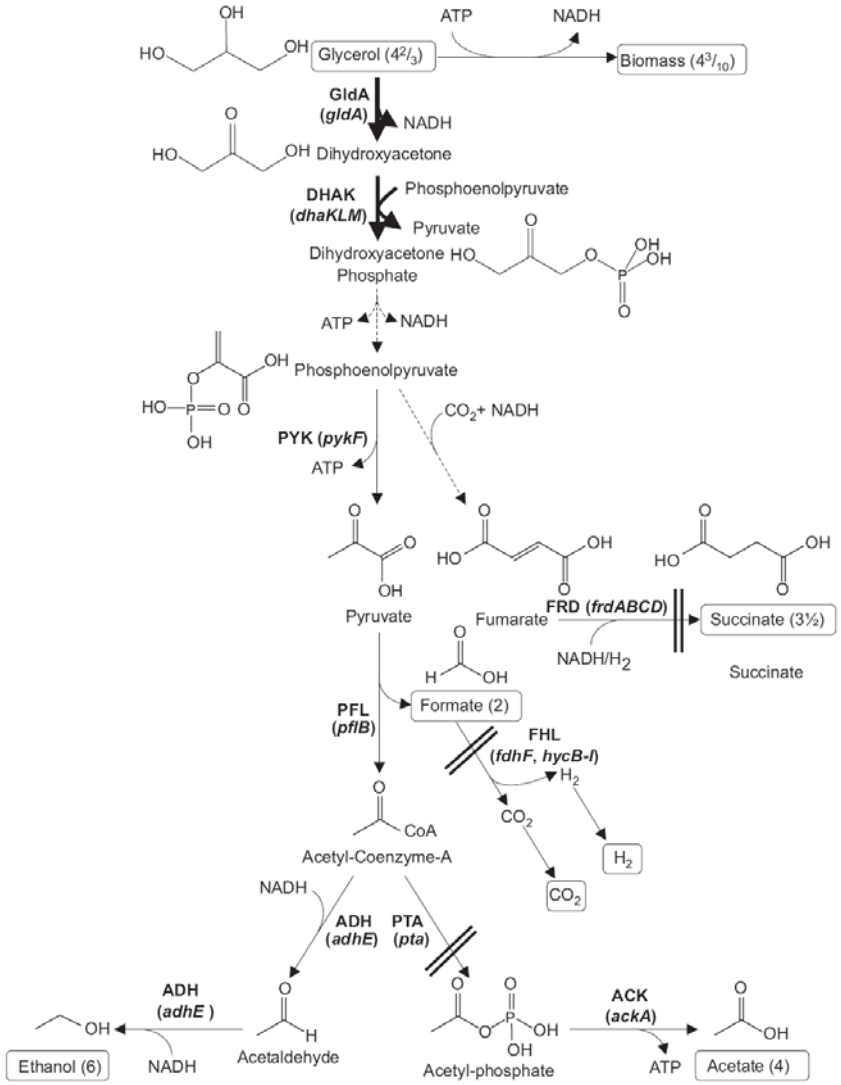


Figure 4.16 Main fermentative pathways involved in the anaerobic fermentation of glycerol in E. coli [33]

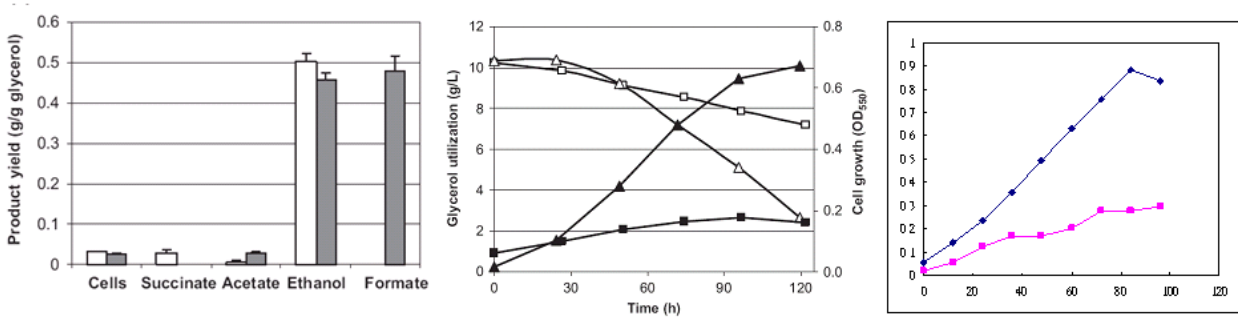


Figure 4.17 Left is performance of strains gay bar represent FHL knock and white bar represent not. Center is Cell growth (close)and glycerol utilization(open), triangle represent FHL knock and square represent not [33]. Right is biomass simulation of FHL deletion :pink represent FHL knock ,dark blue represent not.

4.3 Summery

In the final section, we have to verify the model using parameter analysis and coefficient about parameters and EFMs,the result of coefficient showed the influence of each parameters ,and the tendency of blots can also show the subset of EFMs biological means.

Moreover , the literature descript gene engineering about glycerol fermentation in *E.coli*, the experiments for mortify the *E.coli* genes , raise the yield ethanol and glycerol uptake rate.they induce the GldA and DHAK gene that convert glycerol to dihydorxyacetone and dihydorxyacetone to dihydroxuacetone phosphate. Another varying is blocking FRD for convert fumarate to succinate, FHL for convert formate to CO2 and H2, and PTA for convert Acetyl-Coenzyme-A to acetyl-phosphate.

And we simulate this condition, the reaction rate in whole system are decrease when block the FHL gene, corresponding to experimental data are similar.

Chapter 5 Discussions

5.1 Glycerol anaerobic utilization

Many microorganisms are able to utilize glycerol in the condition of external electron acceptors (respiratory metabolism), but few can ferment glycerol. However, these microorganisms have a few pathogenicity and not efficient than *E.coli.*

Previous study of glycerol fermentation in *E.coli* showed glycerol fermentation in *E.coli* need the external electron acceptor.[26]

5.2 Elementary flux mode

Compare with extreme pathways analysis and elementary flux modes, the former represents a subset of elementary modes, and it identifies a minimum set of generating vectors for a biochemical network's convex operating space that were difficult to interpret.[34] The elementary flux modes analysis was accorded to LP optimization functions.[35]

Non-growth conditions, the product convert into product will observe chemical bond energy order from high-enthalpy, low-entropy to low-enthalpy, high-entropy.[9]

5.3 Modeling

Metabolism of cell can be present by elementary flux modes, and the effects of each mode to whole metabolic pathway are weighted by some parameters, the parameters represent every modes property. The parameters identification was important in cell flux determinate, so the strategy to enable the parameters correctly is especially decisive.[9].the method of constrain the parameters such as thermodynamic method is compute the each modes of entropy with substrate and product every reactions.[9]

5.4 Compound influence cell growth and metabolite distributions

The biomass composition corresponds to different growth and different carbon source that can be influence the metabolic flux during elementary flux modes analysis. Cell maintain intracellular operation were cost a few energy such as ATP, therefore the modes not only contain biomass producing modes ,but also include energy producing modes, which mark off by the result of modes are only comprise ATP produce or biomass produce. Besides, the reaction of oxidation and reduction are critical for whole metabolic network, so numbers of modes will drastic change by delete one or two of this reaction.[35]

The co2 and h2 concentration reduce the glycerol fermentation because of the reducing equivalents be topple over by hydrogen and the CO2 will inhibit the FHL catalyzed.[3] Methylglyoxal accumulation are growth toxicity, it is influenced by damage the DNA repair enzymes.[36] During glycerol fermentation in *E.coli*, MG produce form DHAP via MG synthase.[3]

5.5 Future work

Compare to glucose, the ability of importing to cell that glycerol is not efficient. And the glycolysis intermediate metabolite such as fructose -6- phasphate and the transcription factor will control the related gene expression.

The compete model contain gene regulatory network, enzyme concentration and activity, and metabolic pathway for metabolite concentrations, base on this concept, the large ethanol production's *E.coli* will be construct and apply very efficiently.

Chapter 6 Conclusion

Modeling and analysis provide biologists different opinions about metabolic network. The findings in this study highlight the need for researchers to investigate the above issues and analysis for improving glycerol application in *Escherichia coli* especially.

Although the sample size in the study was small, the following recommendations could serve as general principles for researchers who would like to carry out glycerol anaerobic utilization in *Escherichia coli.*

We proposed that tryptone as the resource for biomass growing, so the tryptone's carbon was excluded from metabolites poured out. During the biomass growing, reactions in biological system may generate the redox factor that could improve the glycerol uptake and boost the glycerol dehydrogenase catalyzed reaction for fermentation.

Besides, we obviously found that glycerol fermentation in medium containing tryptone is prone to generate biomass and the experimental data showed the biomass grown most when the glycerol fermentation happened in tryptone added medium.

Moreover, these findings are parallel with previous studies .The FHL (formate hydrogen lyase) gene knocked out reduced the growing of biomass compared with FHL gene complemented strain. The EFMs also reduced a big ratio when FHL gene knocked out. The model showed the tendency of *E.coli* prefers no formate in cell. When FHL gene was knocked out, the formate accumulated and hindered the metabolic pathway.

When comparing the high yields EFMs and EFMs with high coefficient of metabolites concentration, the later one was average yields predominates in *E.coli*. Although the gene modification resulted in relatively high ratio of high yields EFMs, deletion of average yields will decrease growing of biomass in cell.

Reference

1. Yuan, L., et al., *Electrochemical catalytic reforming of oxygenated-organic compounds: a highly efficient method for production of hydrogen from bio-oil.* Chem Commun (Camb), 2008(41): p. 5215-7.
2. Yazdani, S.S. and R. Gonzalez, *Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry.* Curr Opin Biotechnol, 2007. **18**(3): p. 213-9.
3. Dharmadi, Y., A. Murarka, and R. Gonzalez, *Anaerobic fermentation of glycerol by Escherichia coli: a new platform for metabolic engineering.* Biotechnol Bioeng, 2006. **94**(5): p. 821-9.
4. Quastel, J.H. and M. Stephenson, *Further Observations on the Anaerobic Growth of Bacteria.* Biochem J, 1925. **19**(4): p. 660-6.
5. Savageau, M.A., *Michaelis-Menten mechanism reconsidered: implications of fractal kinetics.* J Theor Biol, 1995. **176**(1): p. 115-24.
6. Keseler, I.M., et al., *EcoCyc: a comprehensive database resource for Escherichia coli.* Nucleic Acids Res, 2005. **33**(Database issue): p. D334-7.
7. Leiser, J. and J.J. Blum, *On the analysis of substrate cycles in large metabolic systems.* Cell Biophys, 1987. **11**: p. 123-38.
8. Carlson, R.P., *Decomposition of complex microbial behaviors into resource-based stress responses.* Bioinformatics, 2009. **25**(1): p. 90-7.
9. Wlaschin, A.P., et al., *The fractional contributions of elementary modes to the metabolism of Escherichia coli and their estimation from reaction entropies.* Metab Eng, 2006. **8**(4): p. 338-52.
10. Schuster, S., T. Dandekar, and D.A. Fell, *Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering.* Trends Biotechnol, 1999. **17**(2): p. 53-60.
11. Schwartz, J.M. and M. Kanehisa, *A quadratic programming approach for decomposing steady-state metabolic flux distributions onto elementary modes.* Bioinformatics, 2005. **21 Suppl 2**: p. ii204-5.
12. Jeong, H., et al., *The large-scale organization of metabolic networks.* Nature, 2000. **407**(6804): p. 651-4.
13. Trinh, C.T., P. Unrean, and F. Srienc, *Minimal Escherichia coli cell for the most efficient production of ethanol from hexoses and pentoses.* Appl Environ Microbiol, 2008. **74**(12): p. 3634-43.
14. Tong, I.T., H.H. Liao, and D.C. Cameron, *1,3-Propanediol production by Escherichia coli expressing genes from the Klebsiella pneumoniae dha regulon.* Appl Environ

Microbiol, 1991. **57**(12): p. 3541-6.
15. Zhang, X.M., et al., *[Construction of novel recombinant Escherichia coli capable of producing 1,3-propanediol].* Sheng Wu Gong Cheng Xue Bao, 2005. **21**(5): p. 743-7.
16. Sprenger, G.A., et al., *Anaerobic growth of Escherichia coli on glycerol by importing genes of the dha regulon from Klebsiella pneumoniae.* J Gen Microbiol, 1989. **135**(5): p. 1255-62.
17. Ahrens, K., et al., *Kinetic, dynamic, and pathway studies of glycerol metabolism by Klebsiella pneumoniae in anaerobic continuous culture: III. Enzymes and fluxes of glycerol dissimilation and 1,3-propanediol formation.* Biotechnol Bioeng, 1998. **59**(5): p. 544-52.
18. Skraly, F.A., B.L. Lytle, and D.C. Cameron, *Construction and characterization of a 1,3-propanediol operon.* Appl Environ Microbiol, 1998. **64**(1): p. 98-105.
19. von Kamp, A. and S. Schuster, *Metatool 5.0: fast and flexible elementary modes analysis.* Bioinformatics, 2006. **22**(15): p. 1930-1.
20. Torres, N.V., and Voit, E.O. , *Pathway Analysis and Optimization in Metabolic Engineering.* Cambridge University Press, Cambridge, U.K 2002.
21. Merriam, *Webster's Medical Dictionary.* 2002.
22. Papin, J.A., et al., *Comparison of network-based pathway analysis methods.* Trends Biotechnol, 2004. **22**(8): p. 400-5.
23. Mendes, P. and D. Kell, *Non-linear optimization of biochemical pathways: applications to metabolic engineering and parameter estimation.* Bioinformatics, 1998. **14**(10): p. 869-83.
24. Stelling, J., et al., *Metabolic network structure determines key aspects of functionality and regulation.* Nature, 2002. **420**(6912): p. 190-3.
25. Kim, J.I., J.D. Varner, and D. Ramkrishna, *A hybrid model of anaerobic E. coli GJT001: combination of elementary flux modes and cybernetic variables.* Biotechnol Prog, 2008. **24**(5): p. 993-1006.
26. Murarka, A., et al., *Fermentative utilization of glycerol by Escherichia coli and its implications for the production of fuels and chemicals.* Appl Environ Microbiol, 2008. **74**(4): p. 1124-35.
27. Rathnasingh, C., et al., *Development and evaluation of efficient recombinant Escherichia coli strains for the production of 3-hydroxypropionic acid from glycerol.* Biotechnol Bioeng, 2009.
28. Sevilla, A., et al., *Model of central and trimethylammonium metabolism for optimizing L-carnitine production by E. coli.* Metab Eng, 2005. **7**(5-6): p. 401-25.
29. Canovas, M., et al., *Role of energetic coenzyme pools in the production of L-carnitine by Escherichia coli.* Metab Eng, 2006. **8**(6): p. 603-18.
30. Guebel, D.V., M. Canovas, and N.V. Torres, *Analysis of the Escherichia coli response to glycerol pulse in continuous, high-cell density culture using a multivariate*

approach. Biotechnol Bioeng, 2009. **102**(3): p. 910-22.

31. Krebs, H.A., *The role of fumarate in the respiration of Bacterium coli commune.* Biochem J, 1937. **31**(11): p. 2095-124.
32. Chhatre, S., et al., *Global Sensitivity Analysis for the determination of parameter importance in bio-manufacturing processes.* Biotechnol Appl Biochem, 2008. **51**(Pt 2): p. 79-90.
33. Shams Yazdani, S. and R. Gonzalez, *Engineering Escherichia coli for the efficient conversion of glycerol to ethanol and co-products.* Metab Eng, 2008. **10**(6): p. 340-51.
34. Schuster, S., et al., *Reaction routes in biochemical reaction systems: algebraic properties, validated calculation procedure and example from nucleotide metabolism.* J Math Biol, 2002. **45**(2): p. 153-81.
35. Carlson, R. and F. Srienc, *Fundamental Escherichia coli biochemical pathways for biomass and energy production: identification of reactions.* Biotechnol Bioeng, 2004. **85**(1): p. 1-19.
36. Ferguson, G.P., D. McLaggan, and I.R. Booth, *Potassium channel activation by glutathione-S-conjugates in Escherichia coli: protection against methylglyoxal is mediated by cytoplasmic acidification.* Mol Microbiol, 1995. **17**(6): p. 1025-33.

Appendix

Table 1 Reaction of elementary flux modes analysis

No.	Reaction
1	GG1 : GLC_ext + PEP = G6P + PYR
2	GG2r : G6P = F6P
3	GG3 : F6P + ATP = F16BP + ADP
4	GG4 : F16BP = F6P
5	GG5r : F16BP = DHAP + GA3P
6	GG6r : GA3P = DHAP
7	GG7r : GA3P + NAD = 3PGP + NADH
8	GG8r : 3PGP + ADP = 3PG + ATP
9	GG9r : 3PG = 2PG
10	GG10r : 2PG = PEP
11	GG11 : PEP + ADP = PYR + ATP
12	GG12 : PYR + ATP = PEP + AMP
13	GG13 : PYR + CoASH + NAD = ACoA + CO2 + NADH
14	PPP1 : G6P + NADP = GL6P + NADPH
15	PPP2 : GL6P = 6PG
16	PPP3 : 6PG + NADP = R5P + CO2 + NADPH
17	PPP4r : R5P = X5P
18	PPP5r : R5P = RIBO5P
19	PPP6r : RIBO5P + X5P = S7P + GA3P
20	PPP7r : GA3P + S7P = ERY4P + F6P
21	PPP8r : ERY4P + X5P = GA3P + F6P
22	TCA1 : OAA + ACoA = CIT + CoASH
23	TCA2r : CIT = CACO
24	TCA3r : CACO = ICIT
25	TCA4 : ICIT + NADP = AKG + CO2 + NADPH
26	FR1 : OAA + NADH = MAL + NAD
27	FR2 : MAL = FUM
28	FR3 : FUM + QH2 = SUCC + Q
29	GLB1 : ICIT = GLYOXY + SUCC
30	GLB2 : GLYOXY + ACoA = MAL + CoASH
31	ANA1 : PEP + CO2 = OAA
32	ANA2 : MAL + NAD = PYR + CO2 + NADH
33	ANA3 : OAA + ATP = PEP + ADP + CO2

34	FEM1 : PYR + CoASH = ACoA + FOR
35	FEM2 : PYR + Q = ACE + CO2 + QH2
36	FEM3 : PYR + NADH = LAC + NAD
37	FEM4 : FOR = CO2 + H2_ext
38	FEM5 : ACoA + NADH = ACA + NAD + CoASH
39	FEM6 : ACA + NADH = ETOH + NAD
40	FEM7 : ACoA = ACP + CoASH
41	FEM8 : ACP + ADP = ACE + ATP
42	FEM9 : PYR = ACA + CO2
43	EDP1 : 6PG = KDPG
44	EDP2 : KDPG = PYR + GA3P
45	XYL1 : XYLO = XYLU
46	XYL2 : XYLU + ATP = X5P + ADP
47	GAL1 : GAL + ATP = G6P + ADP
48	MAN1 : MAN_ext + ATP = MAN6P + ADP
49	MAN2 : MAN6P = F6P
50	ARA1 : ARA + ATP = X5P + ADP
51	BIO : 49 G6P + 17 F6P + 860 RIBO5P + 1426 AKG + 2355 OAA + 512 ERY4P + 960 PEP + 3920 PYR + 1642 3PG + 31 GA3P + 1207 ACoA + 40680 ATP + 4079 NAD + 18320 NADPH + 12502 NH3 = BIOMASS + 1207 CoASH + 40680 ADP + 4079 NADH + 18320 NADP
52	OPM4r : NADH + Q = NAD + QH2
53	OPM3 : ATP = ADP + ATP_main
54	FC1r : NAD + NADPH = NADP + NADH
55	FC2 : AMP + ATP = 2 ADP
56	TRA1 : ETOH = ETOH_ext
57	TRA2 : ACE = ACE_ext
58	TRA3 : NH3_ext = NH3
59	TRA4 : LAC = LAC_ext
60	TRA5 : SUCC = SUCC_ext
61	TRA6 : FOR = FOR_ext
62	TRA7 : CO2 = CO2_ext
63	TRA8 : XYLO_ext + ATP = XYLO + ADP
64	TRA9 : GAL_ext + ATP = GAL + ADP
65	TRA10 : ARA_ext + ATP = ARA + ADP
66	GLYD1 : GLYCEROL_ext = GLYCEROL
67	GLYD2 : GLYCEROL + ATP = G3P + ADP
68	GLYD3 : GLYCEROL + NAD = DHA + NADH
69	GLYD4 : DHA + ATP = DHAP + ADP

70	TRYP : 4 TRYPTONE + 120000 NADH = 3 BIOMASS + 120000 NAD
71	GLYD6 : GLYCEROL = 3HPA
72	GLYD7 : 3HPA + NADH = 13PD + NAD

Table 2 Figures‘s detail EFMs

Figure	Detail EFMs
Figure 2.6	1858: (37) 1.04797 GG4 3.54285 GG11 PPP2 0.0844116 TCA1 0.0844116 TCA2r 0.907776 FR3 0.223816 ANA1 4.31079 FEM1 4.15497 FEM5 4.15497 FEM6 4.31079 FEM4 EDP1 EDP2 6.10352e-005 BIO 0.907776 OPM4r 4.15497 TRA1 0.740051 TRA3 0.907776 TRA5 4.17145 TRA7 5.0556 GLYD1 5.0556 GLYD3 5.0556 GLYD4 -1.00293 GG2r -1.04797 GG5r -4.00763 GG6r 3.92065 GG7r 3.92065 GG8r 3.82349 GG9r 3.82349 GG10r PPP1 -0.0440674 PPP4r 0.0440674 PPP5r -0.00683594 PPP6r -0.00683594 PPP7r -0.0371704 PPP8r 0.0844116 TCA3r 0.0844116 TCA4 irreversible
Figure 2.7	111: (34) GG4 53.0358 GG11 1.7605 TCA1 1.7605 TCA2r 18.9333 FR3 4.66791 ANA1 48.1963 FEM1 44.9457 FEM5 44.9457 FEM6 48.1963 FEM4 0.0012207 BIO 18.9333 OPM4r 44.9457 TRA1 15.4346 TRA3 18.9333 TRA5 45.2889 TRA7 63.7296 GLYD1 63.7296 GLYD3 63.7296 GLYD4 -0.0604858 GG2r -GG5r -62.7296 GG6r 60.9161 GG7r 60.9161 GG8r 58.8889 GG9r 58.8889 GG10r -0.918518 PPP4r 0.918518 PPP5r -0.143188 PPP6r -0.143188 PPP7r -0.77533 PPP8r 1.7605 TCA3r 1.7605 TCA4 -20.8568 FC1r irreversible
Figure 2.8	1274: (40) 1.10956 GG4 1.14355 PPP2 0.143555 PPP3 0.108643 TCA1 0.108643 TCA2r 17.5392 FR1 9.10229 FR2 19.2469 FR3 17.8272 ANA1 8.43683 ANA2 9.13818 FEM1 4.32068 FEM7 4.32068 FEM8 4.61694 FEM5 4.61694 FEM6 9.13818 FEM4 EDP1 EDP2 6.10352e-005 BIO 4.61694 TRA1 4.32068 TRA2 0.952454 TRA3 19.2469 TRA5 19.2469 GLYD1 19.2469 GLYD2 19.2469 GLYD5 -1.14728 GG2r -1.10956 GG5r -18.1374 GG6r 18.0255 GG7r 18.0255 GG8r 17.9003 GG9r 17.9003 GG10r 1.14355 PPP1 0.0390015 PPP4r 0.104553 PPP5r 0.0390015 PPP6r 0.0390015 PPP7r 0.108643 TCA3r 0.108643 TCA4 irreversible
Figure 2.9	64: (33) GG4 53.0358 GG11 1.7605 TCA1 1.7605 TCA2r 4.66791 ANA1 48.1963 FEM1 44.9457 FEM5 44.9457 FEM6 2.90741 FEM4 0.0012207 BIO 44.9457 TRA1 15.4346 TRA3 45.2889 TRA6 82.663 GLYD1 63.7296 GLYD3 63.7296 GLYD4 18.9333 GLYD6 18.9333 GLYD7 -0.0604858 GG2r -GG5r -62.7296 GG6r 60.9161 GG7r 60.9161 GG8r 58.8889 GG9r 58.8889 GG10r -0.918518 PPP4r 0.918518 PPP5r -0.143188 PPP6r -0.143188 PPP7r -0.77533 PPP8r 1.7605 TCA3r 1.7605 TCA4 -20.8568 FC1r irreversible
Figure 2.10	1501: (36) 1.04797 GG4 3.54285 GG11 PPP2 0.0844116 TCA1 0.0844116 TCA2r 4.17145 TRA7 0.223816 ANA1 4.31079 FEM1 4.15497 FEM5 4.15497 FEM6 4.31079 FEM4 EDP1 EDP2 6.10352e-005 BIO 4.15497 TRA1 0.740051 TRA3 5.96338 GLYD1 5.0556 GLYD3 5.0556 GLYD4 0.907776 GLYD6 0.907776 GLYD7 -1.00293 GG2r -1.04797 GG5r -4.00763 GG6r 3.92065 GG7r 3.92065 GG8r 3.82349 GG9r 3.82349 GG10r PPP1 -0.0440674 PPP4r 0.0440674 PPP5r -0.00683594 PPP6r -0.00683594 PPP7r -0.0371704 PPP8r 0.0844116 TCA3r 0.0844116 TCA4 irreversible
Figure 2.11	135: (26) GG4 GG11 3 PPP2 3 PPP3 FEM6 FEM9 TRA1 4 TRA7 2 GLYD1 2 GLYD3 2 GLYD4 6.10352e-005 TRYP -3 GG2r -GG5r -GG6r GG7r GG8r GG9r GG10r 3 PPP1 2 PPP4r PPP5r PPP6r PPP7r PPP8r 6 FC1r irreversible
Figure 2.12	160: (21) GG4 GG11 PPP2 2 FEM6 2 FEM9 EDP1 EDP2 2 TRA1 2 TRA7 2 GLYD1 2 GLYD3 2 GLYD4 -GG2r -GG5r -GG6r GG7r GG8r GG9r GG10r PPP1 FC1r irreversible