การเปลี่ยนกลีเซอรอลเป็นแล็กเทตในระดับหลอดทดลองด้วยเอนไซม์ทนร้อนจากจุลินทรีย์

นางสาวชาลิสา จตุรพักตรารักษ์

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IN VITRO CONVERSION OF GLYCEROL TO LACTATE BY MICROBIAL

THERMOPHILIC ENZYMES

Miss Chalisa Jaturapaktrarak

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By	Miss Chalisa Jaturapaktrarak
Field of Study	Industrial Microbiology
Thesis Advisor	Assistant Professor Suchada Chanprateep Napathorn, Ph.D.
Thesis Co-Advisor	Associate Professor Honda Kohsuke, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

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⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯Thesis Advisor

(Assistant Professor Suchada Chanprateep Napathorn, Ph.D.)

⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯Thesis Co-Advisor

(Associate Professor Honda Kohsuke, Ph.D.)

⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯Examiner

(Assistant Professor Rungaroon Waditee-Sirisattha, Ph.D.)

⋯⋯⋯⋯⋯⋯⋯⋯⋯⋯External Examiner

(Pongsaton Prapakrangkul, Ph.D.)

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การสร้างวิถีเมแทบอลิกจำลองแบบ *in vitro* ถูกริเริ่มนำมาใช้เป็นแนววิธีทางเลือกเพื่อแทน การผลิตชีวภาพจากกระบวนการหมักแบบ *in vivo* โดยเฉพาะอย่างยิ่งการใช้(ไฮเปอร์)เทอร์โมฟิลิ กเอนไซม์ทำให้นักวิจัยสามารถเตรียมไบโอคะตะไลติกโมดูลแบบเลือกเฟ้นที่เสถียรอย่างมากได้ง่าย และการสร้างของวิถีเมแทบอลิกแบบ *in vitro* ในการศึกษานี้ผู้วิจัยออกแบบและสร้างวิถีเมแทบอลิ กจำลองแบบ *in vitro* ซึ่งประกอบรวมด้วย(ไฮเปอร์)เทอร์โมฟิลิกเอนไซม์เก้าชนิด และประยุกต์ใช้ใน การเปลี่ยนกลีเซอรอลไปเป็นแล็กเทต ผู้วิจัยประเมินความเข้ากันได้ของระบบการเปลี่ยนเชิงชีวภาพ แบบ *in vitro* กับเมทานอลซึ่งเป็นสิ่งปนเปื้อนหลักในกลีเซอรอลหยาบที่มาจากกระบวนการผลิตไบ โอดีเซล วิถีจำลองแบบ *in vitro* ได้รับการออกแบบเพื่อทำสมดุลการใช้พลังงานในวิถีภายในและการ สร้างใหม่ของพลังงานและรีด็อกซ์โคแฟกเตอร์ เอมไซม์ทั้งหมดที่เกี่ยวข้องในวิถีแบบ *in vitro* แสดง ระดับที่ยอมรับได้ของความเสถียรที่อุณหภูมิสูง (60°ซ) และความเสถียรนั้นไม่ได้รับผลกระทบอย่าง เห็นได้ชัดที่ความเข้มข้นของเมทานอล 100 mM การเปลี่ยนแบบวัน-พอทของกลีเซอรอลไปเป็นแล็ก เทตผ่านวิถีแบบ *in vitro* สามารถได้รับมาในรูปแบบปริมาณสัมพันธ์ และสามารถผลิตแล็กเทท 14.7 mM ภายใน 7 ชั่วโมง เทอร์โมฟิลิกเอนไซม์จำนวนมากแสดงความเสถียรที่สูงกว่าไม่เพียงแต่ที่ อุณหภูมิสูงแต่ยังรวมถึงที่มีสารแปลงสภาพ ในการศึกษานี้ความเข้ากันได้ของเทอร์โมฟิลิกเอนไซม์กับ เมทานอลได้รับการสาธิตและบ่งชี้การประยุกต์ใช้ที่มีศักยภาพของระบบการเปลี่ยนเชิงชีวภาพแบบ *in vitro* ที่มีเทอร์โมฟิลิกเอนไซม์ในการเปลี่ยนของกลีเซอรอลหยาบไปเป็นสารเคมีที่มีมูลค่าเพิ่ม

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CHALISA JATURAPAKTRARAK: *IN VITRO* CONVERSION OF GLYCEROL TO LACTATE BY MICROBIALTHERMOPHILIC ENZYMES. ADVISOR: ASST. PROF. SUCHADA CHANPRATEEP NAPATHORN, Ph.D., CO-ADVISOR: ASSOC. PROF. HONDA KOHSUKE, Ph.D., 67 pp.

In vitro reconstitution of an artificial metabolic pathway has emerged as an alternative approach to conventional *in vivo* fermentation-based bioproduction. Particularly, employment of (hyper)thermophilic enzymes enables us a simple preparation of highly stable, selective biocatalytic modules and the construction of *in vitro* metabolic pathways. In this study, we designed and constructed an artificial *in vitro* metabolic pathway consisting of nine (hyper)thermophilic enzymes and applied it to the conversion of glycerol to lactate. We also assessed the compatibility of the *in vitro* bioconversion system with methanol, which is a major impurity in crude glycerol released from biodiesel production processes. The *in vitro* artificial pathway was designed to balance the intrapathway consumption and regeneration of energy and redox cofactors. All enzymes involved in the *in vitro* pathway exhibited an acceptable level of stability at high temperature (60°C) and their stability was not markedly affected by the co-existing of up to 100 mM methanol. The one-pot conversion of glycerol to lactate through the *in vitro* pathway could be achieved in an almost stoichiometric manner and 14.7 mM lactate could be produced in 7 h. Many thermophilic enzymes exhibit higher stability not only at high temperatures but also in the presence of denaturants than their mesophilic counterpart. In this study, compatibilities of thermophilic enzymes with methanol were demonstrated, indicating the potential applicability of *in vitro* bioconversion systems with thermophilic enzymes in the conversion of crude glycerol to value-added chemicals.

Department: Microbiology	Student's Signature	....
Field of Study: Industrial Microbiology	Advisor's Signature	～～～～～～～～～
Academic Year: 2014	Co-Advisor's Signature	....

ACKNOWLEDGEMENTS

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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF ABBREVIATIONS

cm	=	Centimeters
°C	=	Degree celcius
g	=	Gram
h	=	Hour
μg	=	Microgram
μl	=	Microliter
mg/g	=	Milligram per gram
mg/L	=	Milligram per litre
mM	=	Millimolar
M	=	Molar
min	=	Minute
mL	=	Millilitre
%	=	Percent
rpm	=	Revolutions per minute
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Statement of the problem

Integration of diverse biocatalytic modules to construct an advanced microbial cell factory has emerged as a powerful approach for the production of industrially important metabolites [1, 2]. Bioprospecting efforts for exploring novel biocatalytic molecules with unique properties have inspired the design and construction of a wider variety of artificial metabolic pathways [3]. However, installation of an artificially engineered metabolic pathway in living organisms often leads to competition with natural metabolic pathways for intermediates and cofactors, resulting in insufficient yield of desired metabolites. A possible solution to this problem is to avoid the use of living microorganisms and to construct an *in vitro* artificial metabolic pathway in which only a limited number of enzymes are involved [4]. Moreover, until now, a variety of *in vitro* synthetic pathways have been designed and constructed for the production of alcohols [2, 5], organic acids [4, 6], carbohydrates [7], hydrogen [8, 9], bioplastic [10] and even electricity [11]. Particularly, employment of enzymes derived from thermophiles and hyperthermophiles enables the simple preparation of catalytic modules with excellent selectivity and thermal stability [6, 12]. Furthermore, although the detailed mechanisms remain to be clarified, many thermophilic enzymes have also been reported to display higher tolerance towards denaturants such as detergents and organic solvents than their mesophilic counterparts [13, 14] and activities of some thermophilic enzymes are even improved with organic solvents [15]. These excellent stabilities of thermophilic enzymes allow great flexibility in the operational conditions of *in vitro* bioconversion systems.

Concerns about the global warming and depletion of fossil fuel reserves have led to the rapid increase of biodiesel production. Generally, 10 kg of crude glycerol, which is the primary byproduct of the biodiesel industry, is released for every 100 kg of biodiesel and the growing production of biodiesel has resulted in a worldwide surplus of crude glycerol [16]. Although many studies have been conducted to use

crude glycerol as a starting material for the fermentation-based production of industrially valuable chemicals, these attempts often suffer from the inhibitory effects of impurities contained in crude glycerol on the growth and biocatalytic activity of living organisms [17, 18]. Particularly, methanol, which is the most abundant impurity in crude glycerol, accounts for up to 70% (w/w) of a raw glycerol obtained through a biodiesel production process [19]. Asad-ur-Rehman et al. reported that a raw glycerol obtained during the biodiesel preparation from sunflower oil contained 50% methanol, which is more abundant than the glycerol content (30%). Lactic acid is naturally presented in many foodstuffs. It is formed by natural fermentation in products such as cheese, yogurt, soy sauce, sourdough, meat products and pickled vegetables and also used in a wide range of food applications such as bakery products, beverages, meat products, confectionery, dairy products, salads and dressings [20]. Lactic acid in food products usually serves as either as a pH regulator or as a preservative [20]. Lactic acid is not only used in food but also pharmaceutical, biodegradable plastics, detergents and animal feed. Therefore, lactic acid reagents are an important industrial material with numerous potential applications [21].

1.2 Objectives

− To construct an *in vitro* artificial pathway for the production of lactate from glycerol using the thermostable biocatalytic modules.

− To demonstrate the capability of thermophilic enzymes with methanol.

1.3 Scope of the research

− To investigate suitable thermophilic enzymes and to construct the *in vitro* artificial metabolic pathway for the conversion of glycerol to lactate.

− To determine enzyme stability against high temperature and high methanol concentration.

− To investigate optimal conditions for the reaction and enzyme level.

— To demonstrate *in vitro* lactate production from glycerol.

1.4 Benefit of research

The potential applicability of *in vitro* bioconversion systems with bacterial thermophilic enzymes in the conversion of glycerol to lactate.

CAPTER II

LITERATURE REVIEW

2.1 Lactic acid

Lactic acid, is a naturally occurring chiral organic compound that has been used in both the food and non-food industries. It is approved by the U.S. Food and Drug Administration as GRAS (generally regarded as safe), so its applications in food and other chemical industries are diverse such as in a bakery products, beverages, meat products, confectionery, dairy products and salads [22]. Lactic acid in food products usually serves as either as a pH regulator or as a preservative [20]. Furthermore, lactic acid has gained enormous demand as feedstock for the chemical synthesis of poly-lactic acid (PLA), a biodegradable polymer, which can be utilized as an environmentally friendly alternative to petro-chemically derived plastics in the textile, medical, pharmaceutical industries and cosmetic industries [22]. The current market price for 88% food grade lactic acid is $1,400–1,600 per metric ton with an annual demand of 130,000–150,000 metric tons, which is estimated to reach 367,300 metric tons by 2017 and over one million tons by 2020 [22].

Lactic acid is produced by lactic acid bacteria as a primary metabolic end-product, such as various *Lactobacillus* species [23]. Conversely, microbial lactic acid fermentation offers an advantage in terms of the utilization of renewable carbohydrate biomass, low production temperature, low energy consumption and the production of optically high pure lactic acid by selecting an appropriate strain [24]. In solution, lactic acid can lose a proton from the carboxyl group ($CH_3CH(OH)CO_2H \longrightarrow H^+ + CH_3CH(OH)CO_2^-$). The biotechnological production of lactic acid requires cheap and renewable raw materials as substrate and other industrial users require large quantities of lactic acid at a relatively low cost, not only produce from glucose but also waste of manufacturing process in industrial factory.

2.1.1 Lactate production

Dien et al. [25] constructed a recombinant *E. coli* for the conversion of glucose as well as pentose sugars into L-lactic acid. Three *E. coli* strains were screened for lactic acid production. Two are derived from K12 strains and the other from a B strain. All three strains were tested for lactic acid fermentation of glucose to lactate. As a result, the fermentation performances of FBR9, FBR10, and FBR11 were compared in pH - controlled batch fermentations with medium containing 10% w / v glucose and batch fermentation results were superior for FBR11, an *E. coli* B strain, compared to those observed for FBR9 or FBR10. FBR11 exhausted the glucose within 30 h and the maximum lactic acid concentration (7.32% w/v) was 93% of the theoretical maximum. The other side - products detected were cell mass and succinic acid (0.5 g/l).

Zhu e al. [26] reported the homofermentative production of lactate in *E. coli* strains containing mutations in the *aceEF*, *pfl*, *poxB* and *pps* genes, which encode the pyruvate dehydrogenase complex, pyruvate formatelyase, pyruvate oxidase and phosphoenolpyruvate synthase, respectively. As a result, the process with a defined medium and two distinct fermentation phases was employed: aerobic growth to an optical density of about 30, followed by nongrowth, anaerobic production. Strain YYC202 generated 90 g/liter lactate in 16 h during the anaerobic phase (with a yield of 0.95 g/g and a productivity of 5.6 g/l·h).

Zhao et al. [27] presented a thermophilic *Bacillus* sp. strain 2–6 was used in completely open repeated batch fermentation for producing optically pure L-lactic acid. When cell growth reached its maximum value of 32 at 16 h, the amount of L-lactic acid produced was 80 g/l. As the cell density decreased from its maximum value to 24 at 37 h, the L-lactic acid concentration increased to 107 g/l.

Masumdar et al. [28] reported the pathways mediating the metabolism of glycerol in *E. coli* under anaerobic and microaerobic conditions. They engineered *E. coli* for the efficient conversion of glycerol to D-lactic acid (D-lactate), a negligible product of glycerol metabolism in wild-type strains. A homofermentative route was engineered by overexpressing pathways involved in the conversion of glycerol to this

product and blocking those leading to the synthesis of competing by-products. The former included the overexpression of the enzymes involved in the conversion of glycerol to glycolytic intermediates (GlpK-GlpD and GldA-DHAK pathways) and the synthesis of D-lactate from pyruvate (D-lactate dehydrogenase). A mutation that blocked the aerobic D-lactate dehydrogenase (Δdld) also was introduced in both LA01 (fumarate reductase (ΔfrdA)) and LA02 (alcohol/acetaldehyde dehydrogenase (ΔadhE)) to prevent the utilization of D-lactate. The most efficient strain (LA02Δdld, with GlpK-GlpD overexpressed) produced 32 g/l of D-lactate from 40 g/l of glycerol at a yield of 85% of the theoretical maximum and with a chiral purity higher than 99.9%. This strain exhibited maximum volumetric and specific productivities for D-lactate production of 1.5 g/l/h and 1.25 g/g cell mass/h, respectively.

2.2 *In vitro* metabolic engineering

Due to a major concern regarding economic, environmental and social importance, industrial microbiology involves the utilization of microorganisms in the production of a wide range of products, including enzymes, foods, beverages, chemical feed-stocks, fuels and pharmaceuticals. However, the metabolisms of natural microorganisms are not evolved to achieve high production yields, productivity and titer of industrial chemicals. Therefore, the optimization of the metabolic flux of microbial cells by enhancing the expression levels of desired genes and/or by depleting those of undesired one has emerged as a powerful strategy to improve microbial cells, the concept so-called "metabolic engineering" [4]. At the beginning, living whole-cell microorganisms were utilized for making several fermentative products for thousands of years. Later, whole cell lysates have been used as an important scientific tool to investigate complicated biological reactions for more than 100 years.

Nevertheless, installation of an artificially engineered pathway in living organisms often leads to competition with natural metabolic pathways for intermediates and cofactors, resulting in insufficient yield of the product of interest

[12]. One of the possible strategies to overcome this limitation is to avoid using living microorganisms and utilize only enzyme involved in the synthetic pathway or *in vitro* metabolic engineering consisted of pathway of interest [4]. The biotransformation catalyzed by whole cell, cell extract, and *in vitro* synthesis are shown in Figure 2.1.

Especially, the *in vitro* system can reduce complex cellular networks. Unlike the *in vivo* system, each enzymatic step is linked with others. For example, a relatively simple pathway is involved in six cascade biochemical reactions where each step is consisted of five choices of genes or enzymes. *In vitro* systems would have 30 combinations since each enzymatic step can be easily exchanged by another enzyme. On the other hand *in vivo* systems may have $5^6 = 625$ combinations and are shown in Figure 2.2 [29].
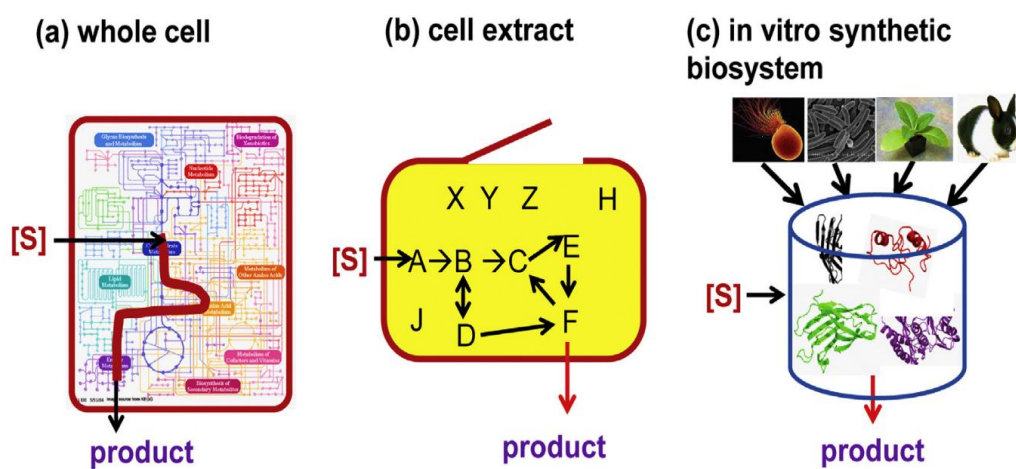
Figure 2.1 Biotransformation catalyzed by (a) whole cell, (b) cell extract, and (c) *in vitro* synthesis biosystem [30].
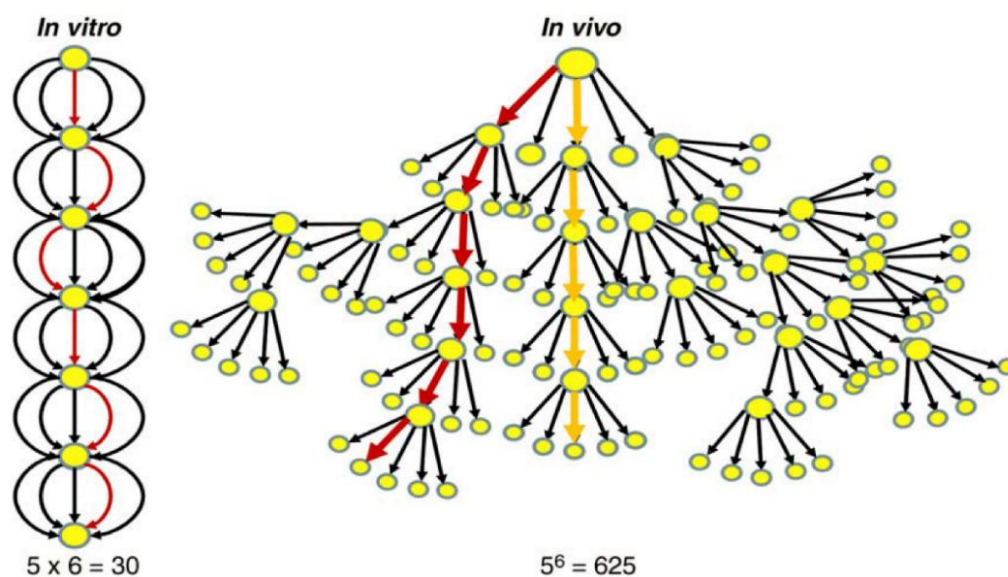
Figure 2.2 Comparision pattern biocatalysis complexity of *in vitro* and *in vivo* systems [29].

2.2.1 Venations in *in vitro* synthesis biosystem

Eduard Buchner discovered the cell-free fermentation for conversion of glucose to ethanol and carbon dioxide using extract of yeast in 1897 [31]. This experiment can be considered as the beginning of the study of *in vitro* systems. Later, he won the Nobel Prize in chemistry in 1907 [32]. Until now, the application of the *in vitro* systems have developed as a part of synthetic biology. There are many kind of products such as biofuel, bioelectricity, alcohol and biodegradable plastic.

2.2.1.1 Biofuel production

Biofuels are energy sources. Their use can significantly reduce greenhouse gas emissions. One of the important biofuel in the future is hydrogen. Zhang et al. [9] demonstrated a synthetic enzymatic pathway consisting of 13 enzymes for producing hydrogen from starch and water, which is inexpensive abundant renewable biomass. The stoichiometry of the reaction through the synthetic pathway can be shown as follows; $C_6H_{10}O_5$ (l) + 7 H_2O (l) $\longrightarrow$ 12 H_2 (g) + 6 CO_2 (g). The yield through the pathway was 12 H_2/glucose, which was higher than the theoretical yield of biological hydrogen

fermentations (4 H_2/glucose) [33, 34]. Moreover, the overall process is spontaneous and unidirectional owing to the negative Gibbs free energy change (ΔG) and the separation of the gaseous product from the aqueous reactants.

Myung et al. [35] constructed a novel *in vitro* non-natural enzymatic pathway comprised of 15 enzymes was designed to convert sucrose to glucose or fructose to high yield hydrogen without the use of costly ATP with a fed-batch reaction was run for enhancing hydrogen generation rates at high substrate concentrations. As a result, when the initial sucrose concentration was 2 mM, dihydrogen evolved as expected. The hydrogen generation rate increased rapidly until 7.2 h. The maximum hydrogen rate was 2.98 mmol/L/ h. When the sucrose concentration was increased from 2 mM to 10 mM at the same enzyme loading, the maximum hydrogen reaction rate was increased by 2.73 fold to 8.14 mmol/L/ h. When the sucrose concentration was increased to 50 mM, the maximum hydrogen generation rate was 9.74 mmol/L/h. Enzymatic hydrogen generation rates based on hexoses have been accelerated from 0.21 mmol/L/h to 9.74 mmol/L/h by nearly 50 fold [36].

The limited supply of fossil resources demands the development of renewable alternatives to petroleum-based products. Biobased higher alcohols such as isobutanol are versatile platform molecules for the synthesis of chemical commodities and fuels. Jan-Karl et al. [5] developed an innovative cell-free approach, utilizing a minimized glycolytic reaction cascade that only requires one single coenzyme. Using this toolbox the cell-free production of ethanol and isobutanol from glucose was achieved. They also confirmed that these streamlined cascades functioned under conditions at which microbial production would have ceased. Measurements indicated that 19.1 mM glucose was converted to 10.3 mM isobutanol within 23 h, which corresponds to a molar yield of 53%. Application of solvent-tolerant biocatalysts potentially allows for high product yields, which significantly simplifies downstream product recovery.

2.2.1.2 Bioelectricity

Enzymatic biofuel cells (EFCs) catalyze oxidation of biomass-based materials for generating electrical energy or electrobiochemical devices that directly convert chemical energy from a variety of fuels into electricity using inexpensive of biocatalyst enzymes [37]. Zhu et al. [11] reported that nearly 24 electrons per glucose unit of maltodextrin can be produced through a synthetic catabolic pathway that comprises 13 enzymes in an air-breathing enzymatic fuel cell. This enzymatic fuel cell is based on non-immobilized enzymes that exhibit a maximum power output of 0.8 mW/cm and a maximum current density of 6 mA/cm^2, which are far higher than the values for systems based on immobilized enzymes. Enzymatic fuel cells containing a 15% (wt/v) maltodextrin solution have an energy-storage density of 596 Ah/kg, which is one order of magnitude higher than that of lithium-ion batteries. Sugar-powered biobatteries could serve as next-generation green power sources, particularly for portable electronics. Zhu et al. [38] also reported that a novel synthetic pathway was designed to perform the deep oxidation of glucose in EFC. Polyphosphate-dependent glucokinase converts glucose to glucose-6-phosphate using low-cost, stable polyphosphate rather than costly ATP. Two NAD-dependent dehydrogenases (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) that were immobilized on the bioanode were responsible for generating two NADH per glucose-6-phosphate (i.e., four electrons were generated per glucose via a diaphorase-vitamin K$_3$ electron shuttle system at the anode). Additionally, to prolong the enzyme lifetime and increase the power output and all of the recombinant enzymes that originated from thermophiles were expressed in *Escherichia coli* and purified to homogeneity. The maximum power density of the EFC with two dehydrogenases was 0.0203 mW/cm^2 in 10 mM glucose at room temperature, which was 32% higher than that of an EFC with one dehydrogenase, suggesting that the deep oxidation of glucose had occurred. When the temperature was increased to 50 °C, the maximum power density increased to 0.322 mW/cm^2, which was approximately eight times higher than that based on mesophilic enzymes at the same temperature.

Xu and Minteer [39] constructed a six-enzyme cascade bioanode containing pyrroloquinoline quinone-dependent enzymes extracted from *Gluconobacter* sp., aldolase from *Sulfolobus solfataricus* and oxalate oxidase from barley to sequentially oxidize glucose to carbon dioxide through a synthetic minimal metabolic pathway. This bioanode is also capable of performing direct electron transfer to carbon electrode surfaces and eliminates the need for mediators. The maximum current density at 0.001 V was 31.5 ± 6.5 $\mu A/cm^2$ for the enzymatic cascade. The maximum power density was 6.74 ± 1.43 $\mu W/cm^2$ for the enzymatic cascade.

2.2.1.3 Biodegradable plastic

Biodegradable plastic can be decomposed by bacteria or other living organisms in soil or environment. It is an environmentally-friendly alternative and does not accumulate in environment. It can replace some amount of the non-degradable plastics, which are produced from petroleum. Opgenorth et al. [10] created a synthetic biochemistry 'purge valve' and integrated it in an enzymatic conversion of pyruvate into polyhydroxybutyrate(PHB). The molecular purge valve could maintain sustainable reducing cofactor balance without the requirement for perfect stoichiometric matching of cofactor generation and carbon usage. They tested the purge valve in the production of PHB bioplastic and isoprene production pathways where cofactor generation and utilization are unbalanced. The full purge valve system produced isoprene an $88.2 \pm 8.4\%$ yield from 3 mM pyruvate. This yield was even higher than the $81.4 \pm 2.0\%$ yield obtained with exogenous addition of NADPH. They found that the regulatory system is highly robust to variations in cofactor levels and readily transportable. The molecular purge valve provides a step towards sustainable biosynthetic systems.

Satoh et al. [40] demonstrated a novel enzyme-catalyzed poly3-hydroxybutryrate P(3HB) synthesis system from acetate that recycles CoA and regenerates NADPH. The system is based on the P(3HB) biosynthesis pathway of *Ralstonia eutropha* and includes P-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase. Furthermore, it contains acetyl-CoA synthetase (Acs) to

produce acetyl-CoA and glucose dehydrogenase (GDH) for the regeneration of NADPH. The weight-average molecular weight of *in vitro* synthesized P(3HB) was 6.64×10^6 g/mol.

2.3 *In vitro* metabolic engineering approach with thermostable biocatalytic modules

Although, the *in vitro* systems could be used for the production of a variety of products, some processes must be considered, such as protein isolation, and simplification of downstream process for product recovery. Recently, Ye et al. [4] have developed an approach to construct *in vitro* artificial metabolic pathways using thermostable biocatalytic modules to overcome these some of limitations.

2.3.1 The basic procedure of methodology

Ye et al. [4] have reported a simple approach to construct an *in vitro* artificial pathway by using thermostable biocatalytic modules, the methodology consisting of 4 steps as shown in Figure 2.3.

I. Selection of the suitable (hyper)thermophilic enzymes.

II. Expression of the enzymes in mesophilic hosts.

III. Preheating the cell suspension at high temperature (Typically, at 70 °C for 30 min).

IV. Rational combination of those catalytic modules at adequate ratio to achieve the stoichiometrical conversion.

Step 1: Selection of thermo-tolerant enzymes and construction of expression vectors

Step 2: Expression in mesophiles (e.g. *E. coli*)

Step 3: Heat-treatment (typically at 70°C for 30 min)

Step 4: Construction of an *in vitro* metabolic pathway with the biocatalytic modules
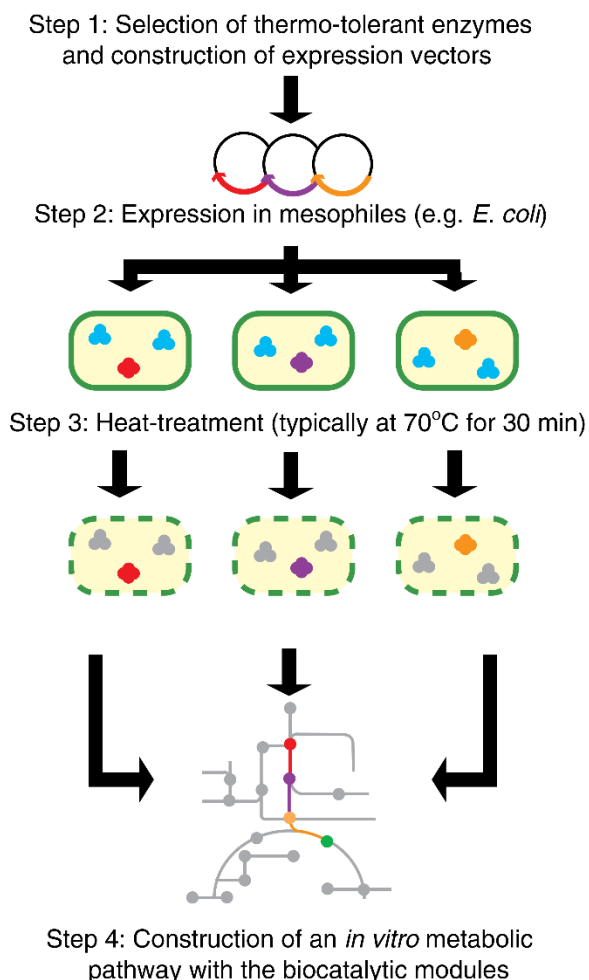
Figure 2.3 The basic procedure for the construction of an *in vitro* artificial pathway by thermostable biocatalytic modules [4].

The *in vitro* synthetic enzymatic pathways are usually designed based on natural metabolic pathways with some modifications, particularly with a careful consideration on cofactor balance, oxygen sensitivity, reaction equilibrium and product separation [30].

For example, stability *in vitro* systems should be designed to balance the production and consumption of ATP whereas living cells can obtain or regenerate ATP from cellular metabolisms. Similar to ATP balance, it is important to design NAD(P)H-balanced pathway. The molar equivalent of reduced NAD(P)H generated from substrates should match with that of NAD(P)H consumption for the production of

desired products [2]. If not, the reaction will less NAD(P)H and finally terminate. Moreover, thermodynamics is also essentially important. It can be calculated from the difference between the free energy of substrates and desired products. For example, butanol production based on glucose is spontaneous (i.e., delta G<0) mainly due to a negative enthalpy [41]. In contrast, methane generation from acetate occurs spontaneously due to a gain of entropy but a positive enthalpy [42]. Synthetic pathways or special enzymatic reactions cannot be accomplished against thermodynamics laws [43]. To ensure driving the reaction towards the desired direction, Gibbs free energy under standard or custom conditions needs to be checked [30].

2.3.2 The conversion of glucose to lactate

Ye et al. [4] have reported a simple approach to construct *in vitro* metabolic pathways so called chimeric Embden-Meyerhof (EM) by using nine recombinant *E. coli* strains overproducing either one of the seven glycolytic enzymes of *Thermus thermophilus*, the cofactor independent phosphoglycerate mutase of *Pyrococcus horikoshii*, or the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase of *Thermococcus kodakarensis* (Figure 2.4). By coupling this pathway with the *Thermus* malate/lactate dehydrogenase, a stoichiometric amount of lactate was produced from glucose. To achieve a balance cofactor consumption and regeneration, they constructed the non-ATP-forming model. In fact, the consumption and regeneration of ATP in a classic EM pathway was not balanced, therefore they designed the chimeric Embden-Meyerhof, in which the enzyme couple of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phophoglycerate kinase in the bacterial EM pathway was replaced with the archaea non-phosphorylating GAPDH (GAPN). As a result, lactate could be produced with a yield of 100%, by 10 h with a feeding of glucose and the final lactate concentration reached was 12 mM.
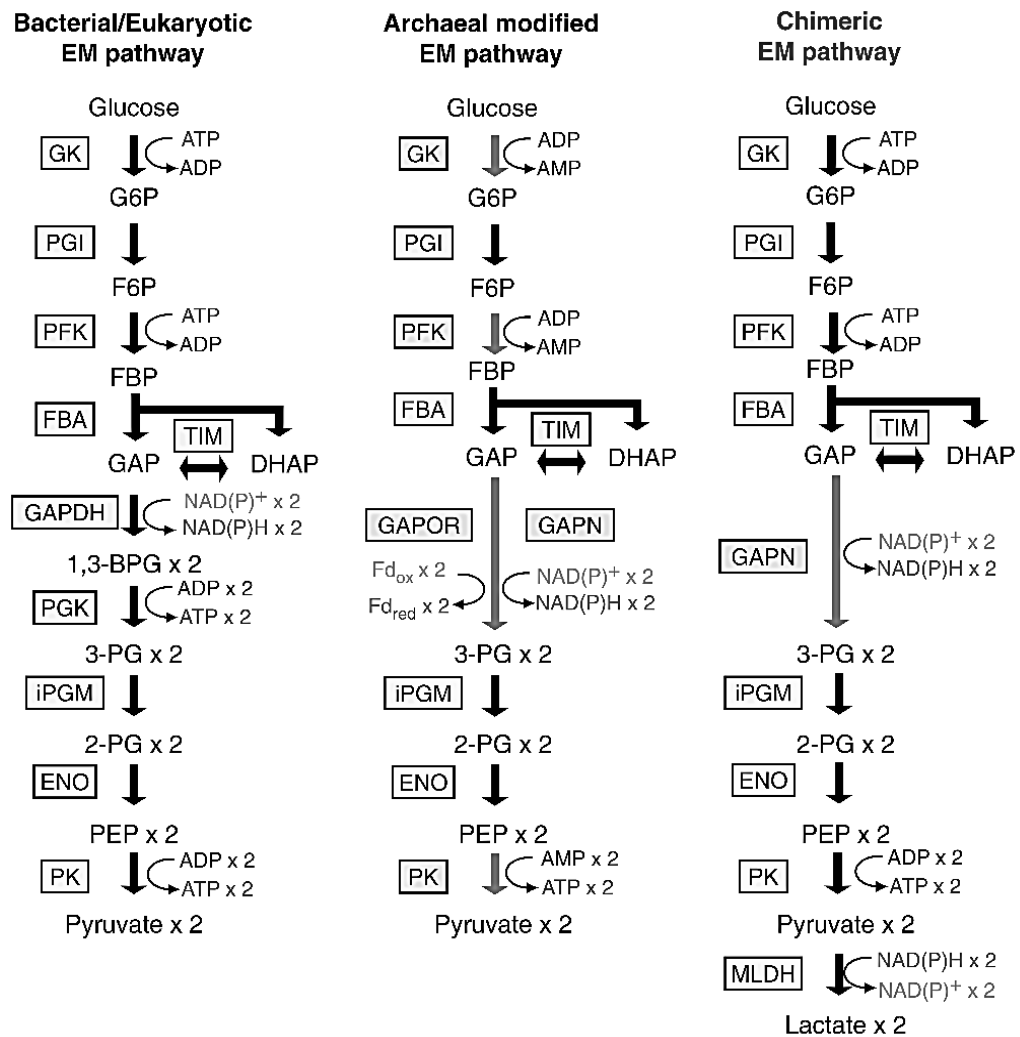
Figure 2.4 The design of chimeric Embden-Meyerhof (EM) pathway [4].

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; GK, glucose kinase; PGI, glucose-6-phosphate isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate aldolase; TIM, triosephosphate isomerase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; MLDH, malate/lactate dehydrogenase, GAPOR, glyceraldehyde-3-phosphate ferredoxin oxidoreductase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; Fdox, oxidized ferredoxin and Fdred, reduced ferredoxin.

2.3.3 The conversion of glucose to malate

In 2013, Ye et al. [6] also demonstrated an *in vitro* artificial pathway for conversion of glucose to malate as shown in Figure 2.5. Using the thermostable biocatalytic modules, they have constructed the chimeric EM pathway combined with another enzyme, namely, the NAD(P)H-dependent malic enzyme derived from a hyperthermophilic archaeon *T. kodakarensis* (TkME) and achieved the direct conversion of glucose to malate (glucose + $2HCO_3^-$ + $2H^+ \longrightarrow 2$ malate + $2H_2O$; ΔG = –121.4 kJ/mol). Malate production was performed at 50 °C using a mixture of heat-treated recombinant cells with experimentally determined amounts of enzymes to achieve a production rate of 0.02 µmol/ml/min. Production rate remained constant during the initial 2 h. Approximately 2.6 mM malate and 0.6 mM lactate were produced from 1.8 mM glucose within 3 h and the malate yield was calculated to be 72%. After 4 h reaction, the molar yield of malate dropped down to 60% because of the thermal decomposition of both $NADP^+$ and NADPH. The depletion of the cofactor seems likely to have caused a decrease in the catalytic performance of GAPN and TkME.
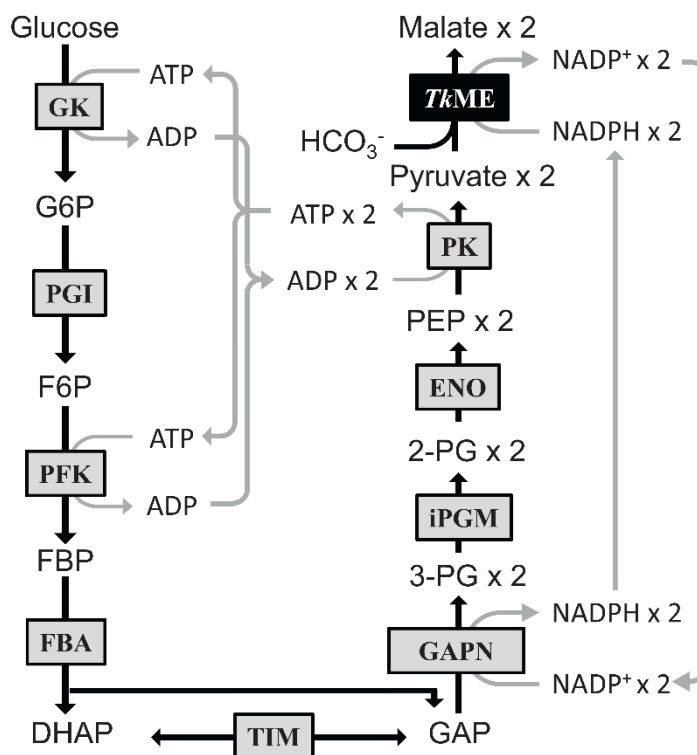
Figure 2.5 The design of synthetic pathway for direct conversion of glucose to malate [6].

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; GK, glucokinase; PGI, glucose-6-phosphate isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate aldolase; TIM, triosephosphate isomerase; iPGM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; ME, malic enzyme and GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase.

2.3.4 The conversion of glucose to n-butanol

Recently, Krutsakorn et al. [2] have constructed a newly designed non-natural, oxygen-insensitive pathway for the direct conversion of glucose to n-butanol, which has increasing interest as a transport fuel by assembling 16 thermotolerant biocatalytic modules. Recombinant *E. coli* strains overproducing the enzymes involved in the

chimeric EM pathway was coupled with thermotolerant enzymes, includging CoA-acylating aldehyde dehydrogenase (TtADDH), acetyl-CoAacetyltransferase (TtACC), hydroxybutyryl-CoAdehydrogenase (TtHBD), enoyl-CoA dehydratase (TtECD) NADH-dependent flavinoxidoreductase (TtNFO) and 3-hydroxyacyl-CoA dehydrogenase (TtHAD) derived from the *Thermus thermophilus* HB8. The pyruvate decarboxylase was derived from *Acetobacter pasteurianus* (ApPDC) and the 3-hydroxypropionyl-CoA dehydratase was derived from *Sulfolobus tokodaii* (StHPD). The pathway was designed to achieve essentially balanced consumption and regeneration rates of ATP and ADP as well as those of NAD^+ and NADH. The metabolic flux through this *in vitro* pathway could be spectrophotometrically monitored in real-time, which enabled to experimentally optimize the level of each enzyme to achieve the desired production rate. The direct conversion of glucose to n-butanol ($C_6H_{12}O_6 \longrightarrow C_4H_9OH+2CO_2+H_2O$; $\Delta G = 282$ kJ/mol) could be achieved in an almost stoichiometric manner. As a result, n-butanol could be produced from glucose with a molar yield of 82% at a rate of 8.2 μmol/ml/min.
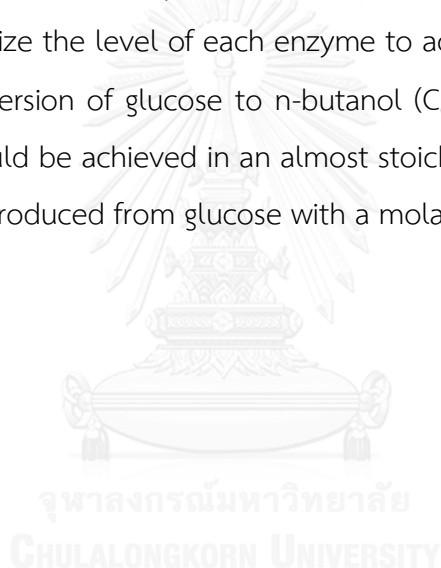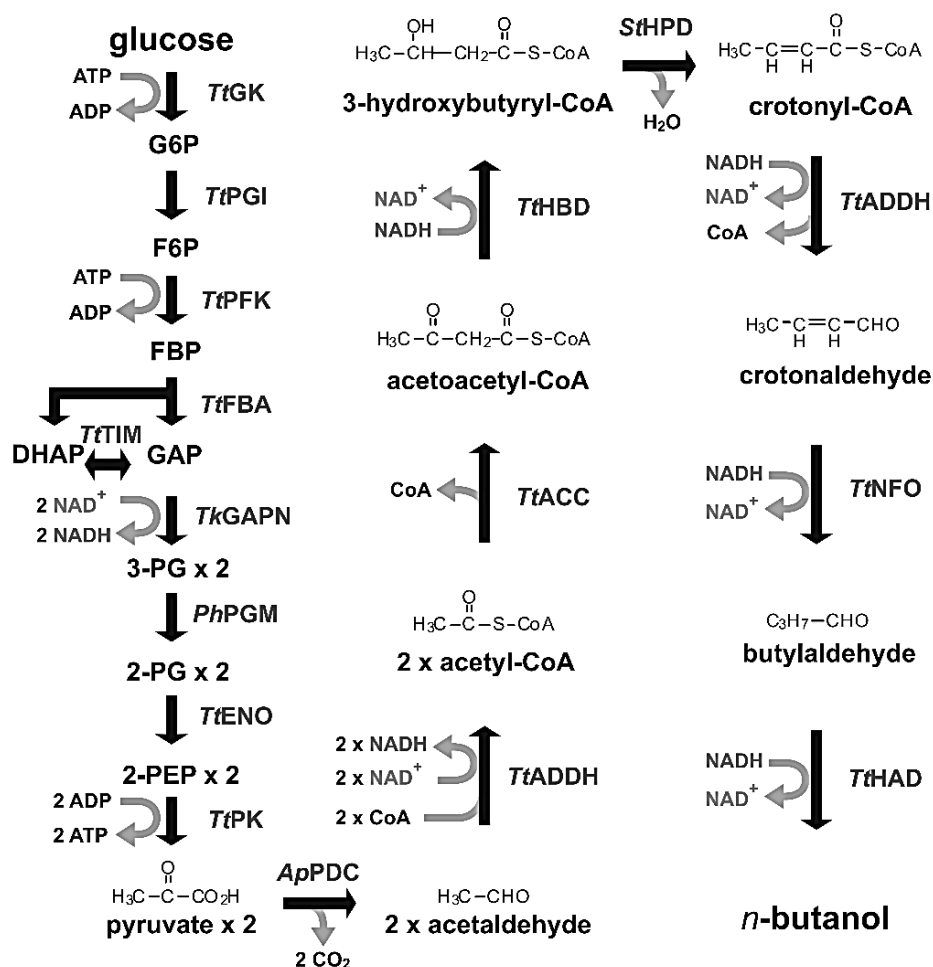
Figure 2.6 The *in vitro* metabolic pathway for conversion of glucose to n-butanol [2].

Abbreviations: G6P,glucose-6-phosphate; F6P,fructose-6-phosphate; FBP,fructose-1,6-bisphosphate; GAP,glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP,phosphoenolpyruvate; TtGK, glucose kinase; TtPGI, glucose-6-phosphate isomerase; TtPFK, 6-phosphofructokinase; TtFBA, fructose-bisphosphate aldolase; TtTIM, triosephosphate isomerase; TkGAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; PhPGM, phosphoglycerate mutase; TtENO, enolase; TtPK, pyruvate kinase; ApPDC, pyruvate decarboxylase; TtADDH, CoA-acylating aldehyde dehydrogenase; TtACC , acetyl-CoAacetyltransferase; TtHBD, hydroxybutyryl-CoAdehydrogenase; StHPD, 3-hydroxypropionyl-CoA dehydratase; TtNFO, NADH-dependent flavinoxidoreductase; TtECD, enoyl-CoA dehydratase and TtHAD, 3-hydroxyacyl-CoA dehydrogenase.

2.4 Benefits of *In vitro* metabolic engineering

Although the metabolic engineering and synthetic biology have been employed for the production of high-value chemicals but has not been as successful in meeting the stringent economics of large-scale commodity chemical manufacturing [10]. Microorganisms have to balance the metabolisms and energy for growth in a culture medium. Therefore, a variety of technical challenges that make it hard to achieve cost competitiveness including, poor yields owing to competing pathway, low productivity caused by slow growth rates or difficulties in pathway optimization, contaminating microbial growth, product toxicity and expensive product isolation [10]. The *in vitro* systems could be overcomed these problems. Implementation of complex biochemical reactions by *in vitro* assembly of multiple metabolic enzymes has several potential advantages, such as elimination of by-product formation, fast reaction rate, thermodynamic prediction of product yield and simplification of downstream processes for product recovery [12].

Particularly, the use of recombinant mesophiles having thermophilic enzymes at high temperatures results in the denaturation of indigenous enzymes and the minimization of unwanted side reactions [4]. Consequently, highly selective thermophilic biocatalytic modules comparable to purified enzymes can be readily obtained without time and cost consuming procedures for enzyme purification. The rational combination of these biocatalytic modules makes it possible to construct *in vitro* synthetic metabolic pathways specific for chemical manufacturing [6]. More importantly, the excellent stability of thermophilic enzymes can mitigate the major disadvantage of *in vitro* enzymatic conversions, namely the inability of protein synthesis and renewal.

2.5 Enzymatic assay with NADH/NAD⁺ oxidation/reduction reactions.

The spectrophotometric assay is the most common detection method in enzyme assays, which is a simple, non-destructive, selective and sensitive. The assay employs a spectrophotometer to measure the amount of substance's absorption, to combine kinetic measurements and calculating the appearance of product or disappearance of substrate concentrations. The NADH/NAD$^+$ molecule is often used in enzymatic oxidation/reduction reactions [44]. The nicotinamide adenine dinucleotide (NAD) exists in two forms, an oxidized and reduced forms abbreviated as NAD$^+$ and NADH respectively. The spectrophotometric enzyme assay with NADH/NAD$^+$ oxidation/reduction reactions is one of the simple way to determine the activities of enzymes in an artificial pathway. The enzyme activities are spectrophotometrically determined by monitoring consumption or generation of NADH at 340 nm (extinction coefficient of 6.22×10^3/M/cm).

In addition, NADH generation can be monitored with the tetrazolium 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) solution as it is reduced in the presence of 1-methoxy PMS (or PMS) and NADH. The assay is also monitored with a spectrophotometer. On reduction, tetrazolium salts produce a dark yellow product called formazan [45]. There has an absorbance at 438 nm and a molar extinction coefficient of 37×10^4/M/cm, which is about 6 times higher than that of NADH at 340 nm The reduction of WST-1 is shown in Figure 2.7.
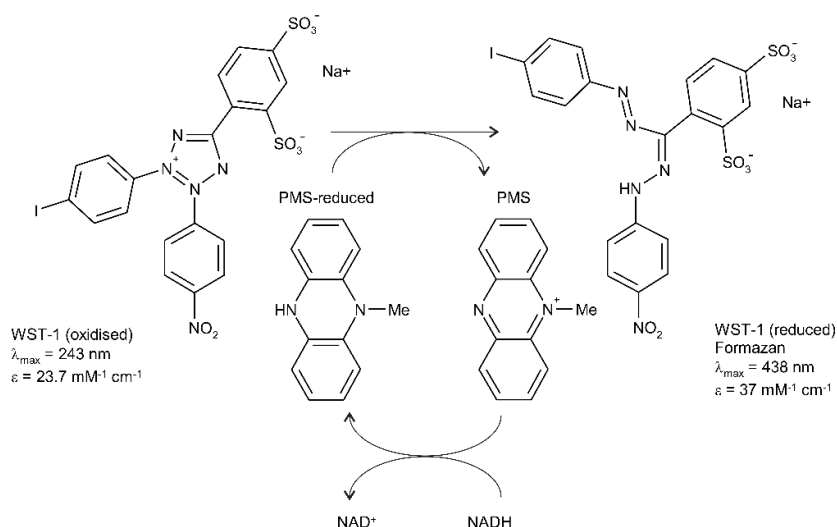
Figure 2.7 The reduction of WST-1 to its reduced form WST-1 formazan by NADH and the intermediate electron acceptor PMS [46].

2.6 Crude glycerol

The biofuel industry is growing tremendously in an effort to reduce the dependency on petroleum-based fuels and address environmental concerns associated with their use [17]. Biodiesel, one of the promising alternative and renewable fuels, has been viewed with increasing interest and its production capacity has been well developed in recent years [18]. Glycerol (also known as glycerin) is a major byproduct in the biodiesel manufacturing process. The biodiesel production on a large commercial scale has led to the accumulation of surplus raw glycerol in world markets. With the production of 10 kg biodiesel, 1 kg glycerol is produced [19]. Glycerol is a valuable raw material for the production of industrially useful metabolites. Among many promising applications for the use of glycerol are its bioconversion to high value-added compounds, such as 1,3-propanediol (1,3-PD), succinate, ethanol, propionate and hydrogen, through microbial fermentation [47]. Glycerol is inexpensive, abundant and has a its better degree of reduction than sugars, offering the opportunity to obtain reduced chemicals, such as succinate, ethanol, xylitol, propionate and hydrogen, at higher yields than those obtained by using sugars [47].

Crude glycerol obtained through the transesterification reaction of fat and vegetable oils (triglycerides) to produce biodiesel. Moreover the production of crude glycerol follows the increasing biodiesel production [48]. It may be possible approach to use crude glycerol as a new carbon source for the production of industrially useful metabolites in fermentation processes. However, the use of crude glycerol in fermentation is a difficult process because crude glycerol contains many impurities such as methanol, salts, soaps and water as the main contaminants [49]. Crude glycerol is expensive to be purified for use in the food, pharmaceutical or cosmetics industries. Biodiesel producers must seek alternative methods for its disposal [50]. Therefore utilizing crude glycerol as the sole carbon source directly is important and will lower the cost of the substrate when compared with glucose [17].

In this study, we focused on establishment to construct an *in vitro* synthetic pathway for the conversion of glycerol to lactate by using nine recombinant *E. coli* strains over producing nine thermophilic enzymes and its compatibility with methanol, a major impurity in crude glycerol, was evaluated.
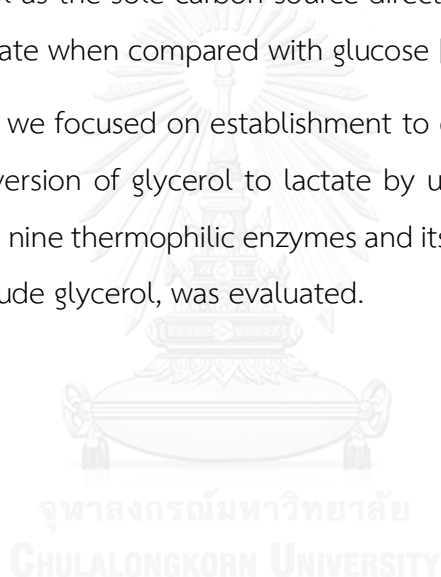
CHAPTER III

MATERIALS AND METHODS

3.1 Analytical instruments

Table 3.1 List of Instruments

Instruments	Model, Manufacturer, Country
Analytical balance	AG-204, Mettler Toledo International Inc, Switzerland
Autoclave	LBS-325, TOMY, Japan
Autopipette	Nichipet Ex, Nichiryo, Japan
High Performance Liquid Chromatography (HPLC)	LC-20 AD, SHIMADZU Corp., Japan
Incubator	SLI-400, EYELA, Japan
Incubator shaker	Multi shaker MMS, EYELA, Japan
Laboratory balance	HF-2000, A&D Company, Japan
Laminar flow cabinet	MCV-13 BNS, SANYO, Japan
Mag-mixer	MA-300, YAMATO, Japan
Microcentrifuge	MX-305, TOMY, Japan
pH meter	F-12, HORIBA, Japan
Spectrophotometer	UV-2450, SHIMADZU Corp., Jpan
Ultrasonic Cleaner	100D, KAIJO, Japan
Ultrasonicator	UD-201, KUBOTA Corp., Japan
Ultrapure water systems	Direct-Q UV, Merk Millipore, Billerica, MA, USA
Vortex-Genie 2	G560, Scientific Industries, USA
Water bath	TR-1A, AS ONE, Japan
Thermomixer	Thermomixer comfort, Eppendorf, USA

3.2 Chemicals

Table 3.2 Chemicals list

Chemicals	Supplier, Country
adenosine diphosphate	Oriental Yeast Co. Ltd., Japan
adenosine triphosphate	Oriental Yeast Co. Ltd., Japan
ampicillin sodium	Wako Pure Chemical Industries Ltd., Japan
Bio-Rad protein assay kit	Bio-Rad Laboratories Inc., Hercules, CA, USA
2-bis(2-hydroxyethyl)amino-2-hydroxymethyl-1,3-propanediol	Nacalai tesque, Japan
chloramphenicol	Wako Pure Chemical Industries Ltd., Japan
dihydroxyacetone phosphate	Sigma-Aldrich, Japan
ethylenediaminetetraacetic acid	Wako Pure Chemical Industries Ltd., Japan
glyceraldrhyde-3-phosphate	Sigma-Aldrich Japan, Japan
glycerol-3-phosphate	Sigma-Aldrich Japan, Japan
glycerol 99%	Wako Pure Chemical Industries Ltd., Japan
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid	Nacalai tesque, Japan

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt	Dojindo Laboratories, Japan
isopropyl β-D-1-thiogalactopyranoside	Nacalai tesque, Japan
lactic acid	Nacalai tesque, Japan
manganese chloride	Wako Pure Chemical Industries Ltd., Japan
magnesium chloride	Wako Pure Chemical Industries Ltd., Japan
methanol	Wako Pure Chemical Industries Ltd., Japan
1-methoxy-5-methylphenazinium methylsulfate	Dojindo Laboratories, Japan
nicotinamide adenine dinucleotide sodium salt	Oriental Yeast Co. Ltd., Japan
nicotinamide adenine dinucleotide, reduced dipotassium salt	Oriental Yeast Co. Ltd., Japan
2-phosphoglycerate	Sigma-Aldrich Japan, Japan
3-phosphoglycerate	Sigma-Aldrich Japan, Japan
phosphoenol pyruvate	Sigma-Aldrich Japan, Japan
pyruvate	Sigma-Aldrich Japan, Japan
sodium hydroxide	Wako Pure Chemical Industries Ltd., Japan
Þ-toluenesulfonic acid	Wako Pure Chemical Industries Ltd., Japan

3.3 Microorganisms and plasmids

Table 3.3 Microorganisms and plasmids used in this study

Strains and plasmids	Description	Reference/ Manufacturer, Country
E. coli JM109	F′(traD36, *pro*AB⁺lacI�q, Δ(lacZ)M15) endA1 recA1 hsdR17(r_k^-,m_k^+) mcrAsupE44λ⁻ gyrA96 relA1 Δ(lac- proAB) thi-1	Sigma-Aldrich Japan, Japan
E. coli Rosetta 2 (DE3)	F⁻*ompT hsdS_B*(r_B^- m_B^-) *gal dcm* (DE3) pRARE2[3] (Camᴿ)	Novagen, Germany
pET-iPGM_Ph	The expression vector encoding cofactor-indepentdent phosphoglycerate mutase of *P. horikoshii* OT3	Ye et al. [4]
pET-GAPN_Tk	The expression vector encoding non-phosphorylating GAP dehydrogenase of *T. kodakarensis*	Ye et al. [4]
pET-GK_Tk	The expression vector encoding glycerol kinase of *T. kodakarensis*	Dr Y. Koga Osaka University [51]
pET-NOX_Tp	The expression vector encoding NADH oxidase of *T. profundus*	Ninh et al. [12]
pET-ENO_Tt	The expression vector encoding enolase of *T. thermophilus* H8	Ye et al., 2013
pET-G3PDH_Tt	The expression vector encoding putative glycerol-3 -phosphate dehydrogenase of *T. thermophilus* H8	The RIKEN *T. thermophilus* HB8 expression plasmid set [52]

pET-LDH$_{Tt}$	The expression vector encoding lactate dehydrogenase of *T. thermophilus* H8	Ye et al. [4]
pET-PK$_{Tt}$	The expression vector encoding pyruvate kinase of *T. thermophilus* H8	Ye et al. [4]
pET-TIM$_{Tt}$	The expression vector encoding triose phosphate isomerase of *T. thermophilus* H8	Ye et al. [4]

3.4 Selection of the suitable thermophilic enzymes and construction of an artificial *in vitro* metabolic pathway for the conversion of glycerol to lactate

The lactate production pathway was designed and the enzyme were selected by using data base including Kyoto Encyclopedia of Genes and Genomes (KEGG), BRENDA and BLAST. The *in vitro* pathway consisting of nine thermophilic and hyperthermophilic enzymes were designed to balance the intrapathway consumption and regeneration of cofactors. Five thermophilic enzymes were derived from the non ATP-forming chimeric EM pathway [4] and combined with four other thermophilic enzymes. They are glycerol kinase, NADH oxidase [53], glycerol-3-phosphate dehydrogenase and lactate dehydrogenase. The *E. coli* JM109 was used for general cloning purpose. *E. coli* Rosetta 2 (DE3) was used for gene expression. The expression vector encoding the glycerol kinase of *T. kodakarensis* (GK$_{Tk}$, gi|3986088) was donated by Dr. Y. Koga, Osaka University [51]. Sources of expression vectors for triose phosphate isomerase (TIM$_{Tt}$, gi|3169211), enolase (ENO$_{Tt}$, gi|55979971), pyruvate kinase (PK$_{Tt}$, gi|55979972), lactate dehydrogenase (LDH$_{Tt}$, gi|55981082) of *T. thermophilus*, non-phosphorylating GAP dehydrogenase of *T. kodakarensis* (GAPN$_{Tk}$, gi|57640640) and cofactor-independent phosphoglycerate mutase of *Pyrococcus horikoshii* (iPGM$_{Ph}$, gi|14589995) were described previously [4]. The expression vector for G3P dehydrogenase of *T. thermophilus* (G3PDH$_{Tt}$, gi|55981709) was obtained from the Riken *T. thermophilus* HB8 expression plasmid set [52]. Gene encoding NADH oxidase of *T.*

profundus (NOX$_{Tp}$, gi|187453160) was cloned and expressed in *E. coli* as described elsewhere [12].

3.5. The enzyme stability at high temperature and high methanol concentration

3.5.1 Preparation of enzyme solution

Recombinants *E. coli* were aerobically cultivated at 37 °C in 5 ml of Luria-Bertani medium (LB) supplemented (Appendix 1) with 100 μg/ml ampicillin and 34 μg /ml chloramphenicol for overnight. After that, 1% (v/v) of cell suspension was transferred in 200 ml of LB supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Gene expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at the late log phase. *E. coli* cells were collected by centrifugation, resuspended in 50 mM HEPES-NaOH (pH 7) and then disrupted by ultrasonicator (Kubota UD-201, Kubota Corp., Osaka, Japan). After the removal of cell debris by centrifugation at 8,000 × g for 5 min, the cell-free extract was incubated at 70 °C for 30 min. The heat-precipitate proteins were removed by centrifugation 15,000 × rpm for 5 min and the resulting supernatant was used as an enzyme solution.

3.5.2 SDS-PAGE and Protein determination

Each of cell lysate and supernatant was used to analyze the expression level of each enzymes by SDS-PAGE (Appendix 2). Standard protocol using 12.5% polyacrylamide gel was employed and the gel was stained with Coomassie brilliant blue R-250 (CBB). The molecular mass of protein was determined by comparing with protein molecular weight marker (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The samples (20 µl) was added to eppendorf tube. The protein assay solution was diluted 5 fold. After that, 1 ml of protein assay solution was mixed with the enzyme solution and incubated at room temperature for 10 min. The absorbance was measured at 595 nm by spectrophotometer. A standard curve was made using the standard solution of bovine serum albumin (2 mg/ml) and concentration of sample protein was calculated from the standard curve (Appendix 3).

3.5.3 Enzymatic assay

Enzyme activities was spectrophotometrically determined at 60 °C by monitoring consumption or generation of NADH at 340 nm using a UV-2450 spectrophotometer. When necessary, NADH generation was coupled with the reduction of WST-1 (0.15 mM WST-1 and 6 µM 1-methoxy PMS) and detected at 438 nm.

GK_{Tk} activity was determined by coupling with $G3PDH_{Tt}$. The standard assay mixture for GK_{Tk} was composed of 50 mM HEPES-NaOH (pH 7), 0.2 mM ATP, 1 mM NAD^+, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, an excess amount of $G3PDH_{Tt}$ and an appropriate amount of GK_{Tk}. The mixture without glycerol was pre-incubated at 60 °C for 2 min and the reaction was initiated by the addition of the substrate (0.2 mM glycerol). $G3PDH_{Tt}$ assay was performed in the same manner except that 0.2 mM G3P was used as a substrate.

TIM$_{Tt}$ was assayed in a mixture containing 50 mM HEPES-NaOH (pH 7), 1 mM NAD$^+$, 5 mM MgCl$_2$, 0.5 mM MnCl$_2$,1 mM glucose-1-phosphate (G1P), an excess amount of GAPN$_{Tk}$ and an appropriate amount of TIM$_{Tt}$. After a pre-incubation at 60 °C for 2 min, the substrate (0.2 mM DHAP) was added to the mixture. For the determination of GAPN$_{Tk}$ activity, 0.2 mM GAP was used instead of DHAP.

Similarly, iPGM$_{Ph}$ activity was assessed by coupling with ENO$_{Tt}$, PK$_{Tt}$ and LDH$_{Tt}$. The enzyme was assayed in a mixture containing 50 mM HEPES-NaOH (pH 7), 0.2 mM ADP, 0.2 mM NADH, 5 mM MgCl$_2$, 0.5 mM MnCl$_2$, appropriate amount of iPGM$_{Ph}$ and excess amounts of ENO$_{Tt}$, PK$_{Tt}$ and LDH$_{Tt}$. the reaction was initiated by the addition of the substrate (0.2 mM 3-PG). The reaction rate was determined by monitoring the concomitant decrease of NADH at 340 nm. Assays for ENO$_{Tt}$, PK$_{Tt}$ and LDH$_{Tt}$ were performed in the same mixture using 0.2 mM each of 2-PG, PEP and pyruvate, respectively.

NOX$_{Tp}$ activity were determined by monitoring the oxidation of NADH under an air atmosphere. A reaction mixture comprising 50 mM HEPES-NaOH (pH 7), 5 mM MgCl$_2$, 0.5 mM MnCl$_2$, 0.02 mM flavin adenine dinucleotide (FAD) and 0.2 mM NADH was preincubated at 60 °C for 2 min. Then, the reaction was started by adding an appropriate amount of enzyme.

One unit of an enzyme was defined as the amount consuming 1 μmol of the substrate per min under the below-mentioned standard assay conditions. The enzyme stability was assessed by measuring the remaining activity of enzymes after the incubation at 60 °C for 8 hour and vary concentration of methanol 0, 50 and 100 mM. The unit of enzyme was calculated by the following equation.

$$\text{Unit/ml enzyme} = \frac{(\Delta A_\lambda/\text{min test} - \Delta A_\lambda/\text{min blank})(V)(df)}{(\epsilon_A)(v)}$$

Where   V   = Total volume of assay (ml)

                λ = 340 nm or 438 nm

                df   = Dilution factor

                ϵ = Molar extinction coefficient at 340 nm = 6.22×10^3/M/cm

                               or Molar extinction coefficient = 37×10^4/M/cm

                v   = Volume of enzyme used (ml)

3.6 The optimal conditions for the reaction and enzyme

3.6.1 Optimization of temperature and pH level

The reaction mixture (1 ml) consisted of 50 mM HEPES-NaOH (pH 7), 0.2 mM glycerol, 1 mM NAD^+, 0.2 mM NADH, 0.2 mM ATP, 0.2 mM ADP, 0.02 mM FAD, 0.5 mM FBP, 1 mM G1P, 5 mM $MgCl_2$ and 0.5 mM $MnCl_2$. GK_{Tk}, $G3PDH_{Tt}$, TIM_{Tt}, $GAPN_{Tk}$, $iPGM_{Ph}$, ENO_{Tt}, PK_{Tt}, LDH_{Tt} and NOX_{Tp} were added to the reaction mixture in 1.5 ml eppendorf tube at a concentration of 0.1 U/ml. The reaction was initiated by the addition of 10 mM glycerol. The reaction mixture was incubated at 50, 60 and 70 °C for 30 min. Similarly, then the reaction was carried out at different pH of 6, 7 and 8.

3.6.2 Optimization of enzyme concentration to achieve a desired lactate production rate [4]

For the real-time monitoring of the flux through the *in vitro* pathway, the pathway was divided in to partial pathways, in which the NAD(H)-dependent enzymes were assigned to be the last step and the consumption and regeneration of NADH was monitored (Appendix 4). The whole pathway (Fig. 4.1) was divided into the following 3 parts: (1) from glycerol to DHAP, (2) from DHAP to 3-PG and (3) 3-PG to lactate.

The first part was determined by coupling with GK_{Tk} and $G3PDH_{Tt}$. The reaction mixture was composed of 50 mM HEPES-NaOH (pH 7), 0.2 mM glycerol, 0.2 mM ATP, 1

mM NAD$^+$, 5 mM MgCl$_2$, 0.5 mM MnCl$_2$ and an appropriate amount of GK$_{Tk}$ or G3PDH$_{Tt}$. The enzyme was increased by one another until a desired lactate production rate was achieved.

The second part was determined by coupling with TIM$_{Tt}$ and GAPN$_{Tk}$. The reaction mixture was composed of 50 mM HEPES-NaOH (pH 7), 0.2 mM DHAP, 1 mM NAD$^+$, 5 mM MgCl$_2$, 0.5 mM MnCl$_2$,1 mM glucose-1-phosphate (G1P) and an appropriate amount of TIM$_{Tt}$ or GAPN$_{Tk}$.

The third part was determined by coupling with iPGM$_{Ph}$, ENO$_{Tt}$, PK$_{Tt}$ and LDH$_{Tt}$. The reaction was composed of 50 mM HEPES-NaOH (pH 7), 0.2 mM 3-PG, 0.2 mM ADP, 0.2 mM NADH, 5 mM MgCl$_2$, 0.5 mM MnCl$_2$ and an appropriate amount of ENO$_{Tt}$, PK$_{Tt}$ and LDH$_{Tt}$.

The reaction rate was determined by monitoring consumption or generation of NADH at 340 nm using a UV-2450 spectrophotometer (Shimadzu Corp., Kyoto, Japan). When necessary, NADH generation was coupled with the reduction of WST-1 (0.15 mM WST-1 and 6 μM 1-methoxy PMS) and detected at 438 nm.

3.7 The *in vitro* lactate production from glycerol

The reaction mixture (4 ml) consisting of 50 mM HEPES-NaOH (pH 7), 0.2 mM glycerol, 1 mM NAD$^+$, 0.2 mM NADH, 0.2 mM ATP, 0.2 mM ADP, 0.02 mM FAD, 0.5 mM FBP, 1 mM G1P, 5 mM MgCl$_2$ and 0.5 mM MnCl$_2$. Enzymes were added to the reaction mixture to give following final concentrations were shown in Table 4.3. The mixture was prepared in a 10-ml cylindrical vessel and incubated at 60 °C with stirring. Glycerol solution (160 mM) was continuously supplied to the mixture at a flow rate of 1 μl/min using a Shimadzu LC-20 AD solvent delivery unit (Shimadzu Corp., Kyoto, Japan). Aliquots (50μl) of the reaction mixture were sampled at every 1 h for 7 h and diluted four-fold with distilled water. The sample was ultrafiltrated using Amicon 3 K (Merk Milipore, Billerica, MA, USA). Lactate was quantified by High-performance liquid chromatography (HPLC) equipped with two tandemly connected ion exclusion columns (Shim-pack SPR-H, 250 mm × 7.8 mm, Shimadzu Corp., Kyoto, Japan). The

column was eluted at 50 °C using 4 mM p-toluenesulfonic acid as a mobile phase at a flow rate of 0.2 ml/min. The eluent was mixed with a pH-buffered solution (16 mM Bis-Tris, 4 mM p-toluenesulfonic acid and 0.1 mM EDTA) supplied at a flow rate of 0.2 ml/min and then analyzed for lactate using a conductivity detector (CDD-20A, Shimadzu Corp., Kyoto, Japan). In order to assess the compatibility of the *in vitro* system with crude glycerol, a model solution of crude glycerol consisting of 30% (w/v) glycerol and 50% (w/v) methanol was prepared and used as a substrate for lactate production.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Design of the *in vitro* synthetic pathway

The newly designed synthetic pathway for the conversion of glycerol to lactate was shown in Figure 4.1. To construct an *in vitro* synthetic pathway, it is vital to prevent the depletion of energy and redox cofactors (ATP/ADP, and NAD^+/NADH) by balancing their intrapathway consumption and regeneration. In a previous study, constructed an ATP/ADP-balanced chimeric Embden-Meyerhof (EM) pathway was constructed by swapping the enzyme couple of GAP dehydrogenase and phosphoglycerate kinase in the bacterial/eukaryotic EM pathway with the nonphosphorylating GAP dehydrogenase ($GAPN_{Tk}$) involved in the modified EM pathway of a hyperthermophilic archaeon, *T. kodakarensis* [4]. Similarly, in this study, the balancing of consumption and regeneration of ATP and ADP was achieved by employing the GAPN-mediated non-ATP-forming dehydrogenation of GAP to 3-PG through the glycerol converting pathway.

On the other hand, the conversion of one molecule of glycerol to lactate through the designed pathway was accompanied by the generation of one molecule of NADH. To re-oxidize the cofactor and to maintain the redox balance of the whole pathway, a hyperthermophilic NADH oxidase was integrated into the pathway. NADH oxidases catalyze the reduction of O_2 using NAD(P)H as a reductant and can be divided into two groups: those catalyzing two-electron reduction of O_2 to H_2O_2 and those catalyzing four-electron reduction of O_2 to H_2O. In this study, the balancing of consumption and regeneration of NAD^+ and NADH employed the NADH oxidase from *T. profundus* (NOX_{Tp}), which preferably catalyzes four-electron reduction of O_2 [53], to eliminate the inhibitory effects of H_2O_2 on enzymes. The chemical equation of the overall reaction through the synthetic pathway can be shown as follows: $HOCH_2CHOHCH_2OH + 1/2O_2 = CH_3CHOHCOO^- + H_2O + H^+$. The standard Gibbs energy change (ΔG) of the reaction was calculated to be -256.4 kJ/mol.
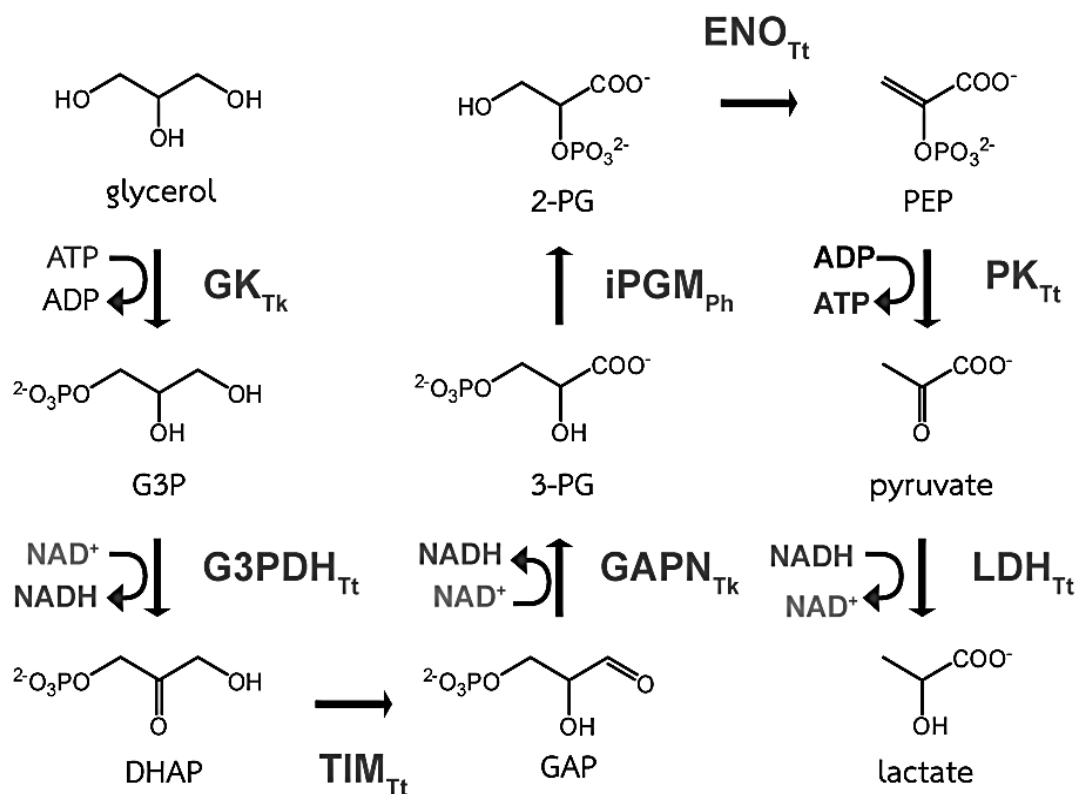
Figure 4.1 Schematic illustration of the *in vitro* synthetic pathway conversion of glycerol to lactate.

4.2 Enzyme stability

4.2.1 Effect of heat treatment on the indigenous proteins of recombinant *E. coli* cells

SDS-PAGE analysis showed that indigenous proteins in the crude extract of *E. coli* cells without expression vector were almost denatured by heat treatment at 70°C for 30 min (Figure 4.2, None). On the other hand, the heat-treated cell-free extract of the recombinant *E. coli* with expression vector showed extra protein bands that were not found in that of *E. coli* without the expression vector (Figure 4.2) as most of host derived proteins were removed by the heat precipitation.
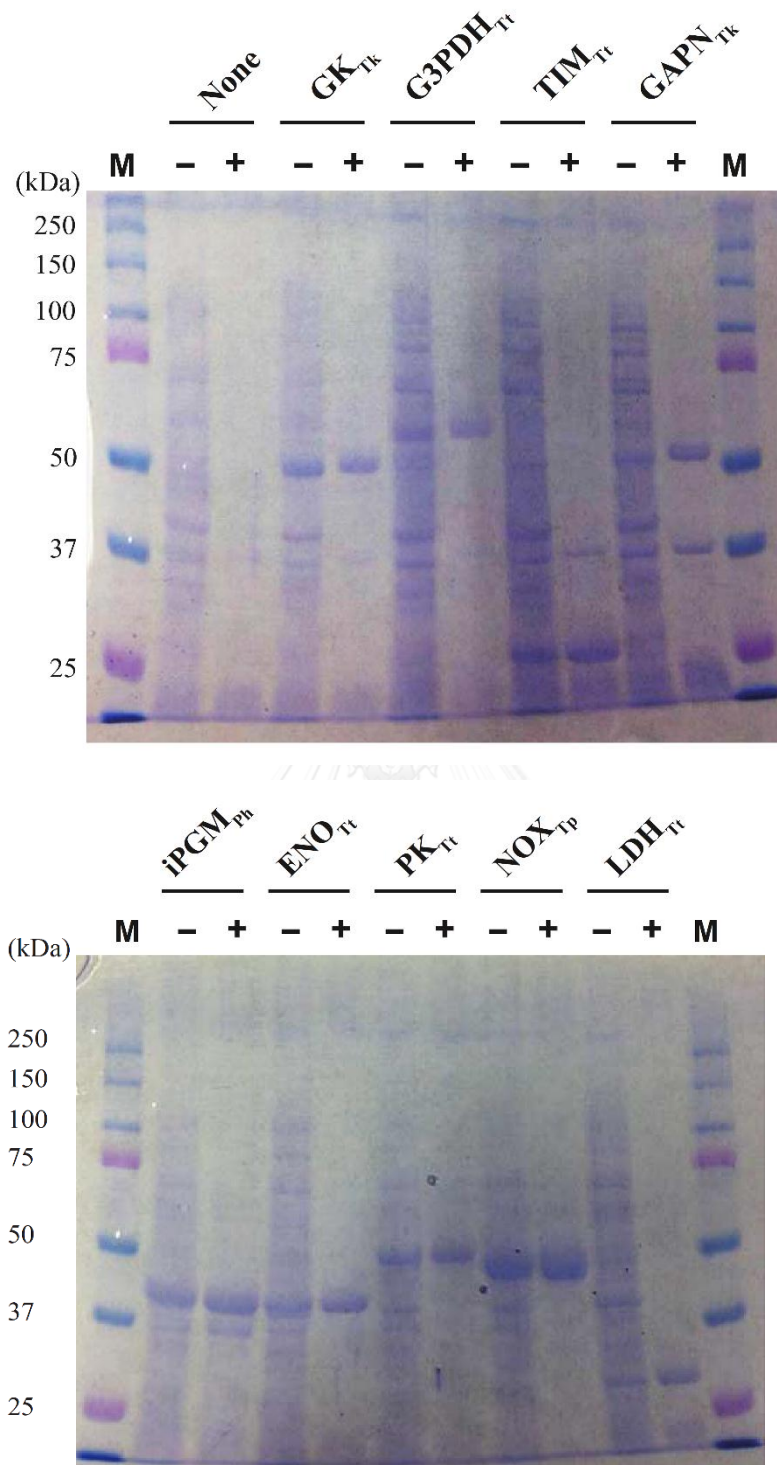
Figure 4.2 The SDS-PAGE analysis of the crude extracts of nine recombinant *E. coli* cells before (-) and after (+) heat treatment at 70°C for 30 min. Samples were from approximately 1 mg (wet weight) of the cells overproducing indicated thermophilic enzymes and separated on 12% acrylamide gels. Dual Xtra prestained protein standard

(Bio-Rad Laboratories Inc.) was used as a protein marker (lanes indicated by M). The calculated molecular mass of recombinant enzymes (kDa) are as follows: GK, 53.4; G3PDH, 56.0; TIM, 27.0; GAPN, 55.5; iPGM, 45.1; ENO, 45.5; PK, 51.1; NOX, 50.0 and LDH, 32.8.

Moreover, while recombinant *E coli* cells before the heat treatment could grow on LB agar, cells after the heat treatment failed to indicating that the *in vitro* system which is free from cell viability (Figure 4.3).
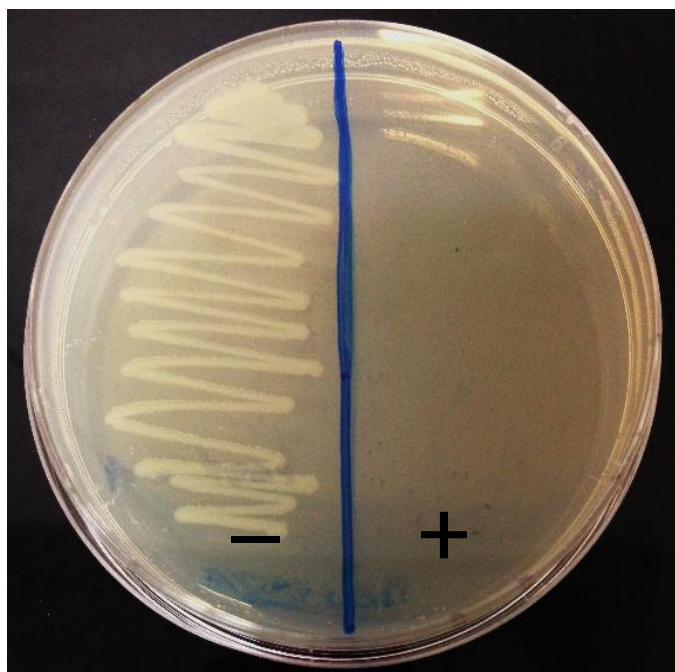
Figure 4.3 The comparison of growth of recombinant *E coli* cells before (-) and after (+) heat treatment at 70°C for 30 min on LB agar.

4.2.2 Effect of thermal stability and methanol

The enzyme stability was assessed by measuring the remaining activity of enzymes after the incubation at 60 °C for 1-8 h. The results showed that most enzymes could retain more than 80% of their initial activity for 8 h, except that PK_{Tt} lost 35% of the activity after the incubation for the same time period (Figure 4.4). The author also assessed the effect of methanol, which is the primary impurity contained in crude glycerol, on the enzyme stability [18]. Although residual activities of TIM_{Tt}, $iPGM_{Ph}$,

ENO_{Tt} and LDH_{Tt} were moderately lower than those in the absence of methanol (16-32% decrease), the destabilization profile of other enzymes were not significantly affected by at least up to 100 mM of methanol. The results were shown in Figure 4.4.
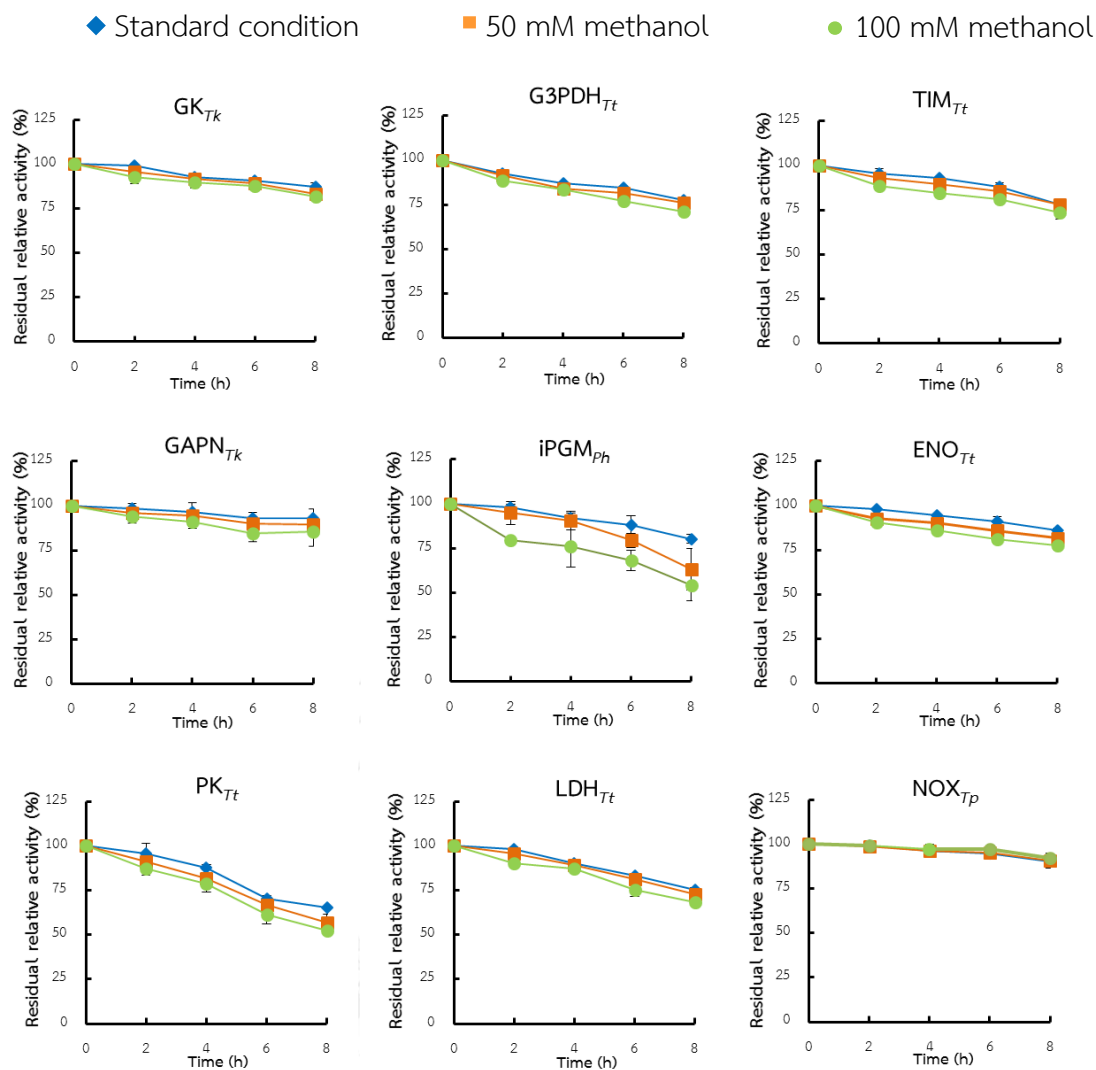
Figure 4.4 Enzyme stability. Enzyme solutions were incubated at 60°C for indicated time periods and residual activities were determined under the standard assay conditions (blue diamond). Enzyme stabilities were also assessed at 60°C in the presence of 50 (orange square) and 100 mM methanol (green circle).

4.3 Optimization of reaction condition

4.3.1 Effect of pH

The lactate production rate through the synthetic pathway was determined at different pH (6-8) by incubating 0.1 U/ml each of GK_{Tt}, $G3PDH_{Tt}$, TIM_{Tt}, $GAPN_{Tk}$, $iPGM_{Ph}$, ENO_{Tt}, PK_{Tt}, LDH_{Tt} and NOX_{Tp} with 10 mM glycerol and appropriate concentrations of cofactors, metal ions and an appropriate buffer. Reaction was performed at 60°C and indicated pH for 30 min and then terminated by removing the enzymes with ultrafiltration. Fifty millimolar of MES-NaOH (pH 6) and HEPES-NaOH (pH 7 and 8) were used to adjust pH. The highest lactate production rate of 0.036 µmol/min/ml was observed in HEPES-NaOH at pH 7 (Figure 4.5).
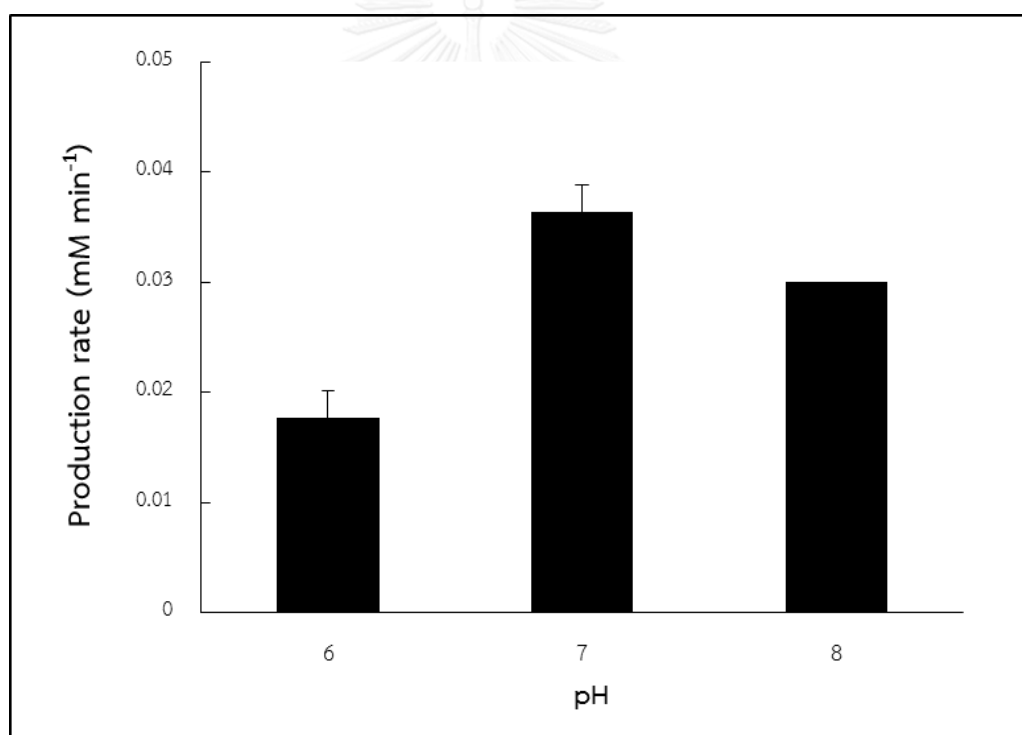
Figure 4.5 Effect of pH on the lactate production through the *in vitro* synthetic pathway.

4.3.2 Effect of temperature

The lactate production rate through the synthetic pathway was determined at different temperature (50-70 °C) by incubating 0.1 U/ml each of GK_{Tt}, $G3PDH_{Tt}$, TIM_{Tt}, $GAPN_{Tk}$, $iPGM_{Ph}$, ENO_{Tt}, PK_{Tt}, LDH_{Tt} and NOX_{Tp} with 10 mM glycerol and appropriate concentrations of cofactors, metal ions. Reaction was performed at indicated temperature for 30 min and then terminated by removing the enzymes with ultrafiltration. The results showed that no significant difference was observed in production rates at 60°C and 70°C ($P>0.1$, Student's t-test). Thus, the reaction temperature of 60°C was employed for further studies to mitigate the thermal inactivation of the enzymes and the decomposition of thermo-labile intermediates and cofactors [4]. The result was shown in Figure 4.6.
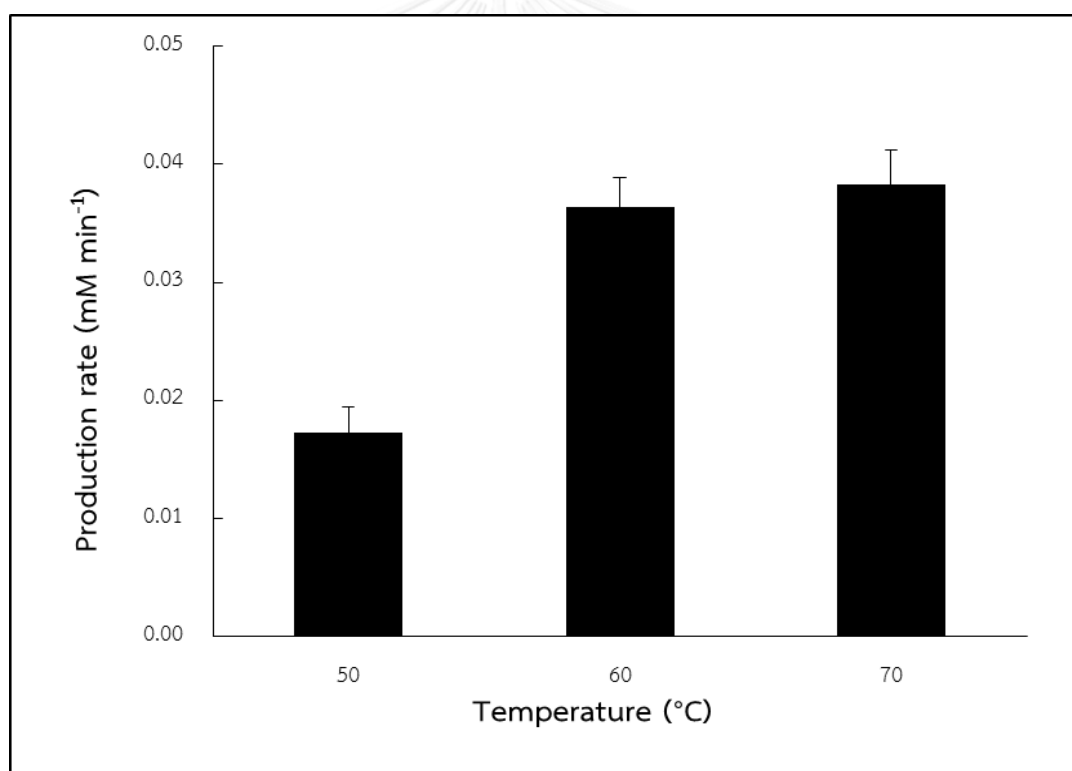
Figure 4.6 Effect of temperature on the lactate production through the *in vitro* synthetic pathway.

4.3.3 Effect NAD$^+$ and FBP on the enzyme activity

Although LDH$_{Tt}$ is allosterically inhibited by NAD$^+$ [4], lactate dehydrogenases can generally be activated by fructose-1,6-bisphosphate (FBP). Enzyme assays were performed with and without 0.5 mM FBP in the reaction mixture containing various concentrations of NAD$^+$. Results were shown as specific enzyme activities, which were assessed using a heat-treated cell-free extract and normalized by the protein concentration of the corresponding non-heated cell-free extract (U/mg protein). The result showed that the LDH$_{Tt}$ activity in the presence of 1 mM NAD$^+$ could be recovered to the similar level to that under the standard assay conditions by the addition of 0.5 mM FBP (Table 4.2).

Table 4.2 The effect of NAD$^+$ and FBP on the activity of LDH$_{Tt}$.

FBP (mM)	NAD$^+$ (mM)			
	0	0.2	0.5	1
0	192 ± 4.20	2.51 ± 0.10	1.08 ± 0.03	0.31 ± 0.02
0.5	1,150 ± 15	980 ± 3.50	396 ± 6.20	230 ± 2.90

4.4 Lactate production

4.4.1 Flux optimization

Unlike highly branched metabolic pathways in living organisms, *in vitro* synthetic pathways, in which only a limited number of enzyme reactions are sequentially aligned, appear to be less sensitive to the imbalance in enzyme concentrations. Although the existence of a rate-limiting enzyme leads to the accumulation of the specific intermediate, it is eventually converted by down-stream enzymes without being routed into the co-existing pathway. However, the accumulation of chemically labile intermediates will result in their spontaneous degradation and decrease in the overall yield of product. Previously, Ye et al. [4]

demonstrated that the flux through an *in vitro* metabolic pathway can be spectrophotometrically determined by dividing the whole pathway into some partial pathways, in each of which the NAD(H)-dependent enzymes are assigned to be the last step and by monitoring the concomitant consumption or production of NAD(P)H through the partial pathways [4]. This real-time monitoring technique enables us to identify rate-limiting enzymes in an *in vitro* pathway by increasing the concentration of each enzyme, one another. The optimum concentrations of enzymes to achieve a desired flux can be experimentally determined by modulating the concentrations of the rate-limiting enzymes [2, 4]. Accordingly, we divided the glycerol converting pathway in three parts, namely from glycerol to DHAP, from DHAP to 3-PG, and from 3-PG to lactate and then adjusted the enzyme concentrations in each partial pathway separately. The reaction rate was derived from the previously experiment of the optimization of reaction condition. Previously, the result showed the highest reaction rate at 0.036 μmol/ml/min under the conditions were pH 7 and 60 °C then set the reaction rate to 0.04 μmol/ml/min for determined flux optimization. As a result, the optimum enzyme concentrations to achieve a lactate production rate of 0.04 μmol/ml/min were determined as shown in 4.3. Accordingly, NOX_{Tp} was put in the reaction mixture to give a NADH re-oxidizing rate of 0.04 μmol/ml/min.

Table 4.3 The unit of enzymes to achieve a lactate production rate of 0.04 µmol/ml/min.

Enzymes	Unit/ml
GK$_{Tt}$	0.04
G3PDH$_{Tt}$	0.18
TIM$_{Tt}$	0.04
GAPN$_{Tk}$	0.10
iPGEM$_{Ph}$	0.09
ENO$_{Tt}$	0.07
PK$_{Tt}$	0.09
LDH$_{Tt}$	0.08
NOX$_{Tp}$	0.04

4.4.2 Effect of thermal decomposition of NAD$^+$ and NADH

Although the synthetic pathway was designed to achieve the balanced reduction and oxidation of NAD$^+$ and NADH, the thermal decomposition of the cofactors was not negligible. Moreover, the rate of the GAPN$_{Tk}$ mediated reaction is sensitive to the depletion of the cofactors owing to its high Km for NAD$^+$ [54]. In this study, 1mM NAD$^+$ and 0.2 mM NADH was incubated at 60°C in 50 mM HEPES-NaOH (pH 7.0). After the incubation for indicated time periods, residual concentrations of the cofactors were determined by HPLC as described elsewhere [12]. Data represent the averages of triplicate assays. The result showed that thermal decomposition rate of NAD$^+$ and NADH were 0.001 µmol/ml/min. Owing to this fact, NAD$^+$ was also continuously supplied to the reaction mixture at a rate identical to that of its thermal decomposition 0.001 µmol/ml/min.
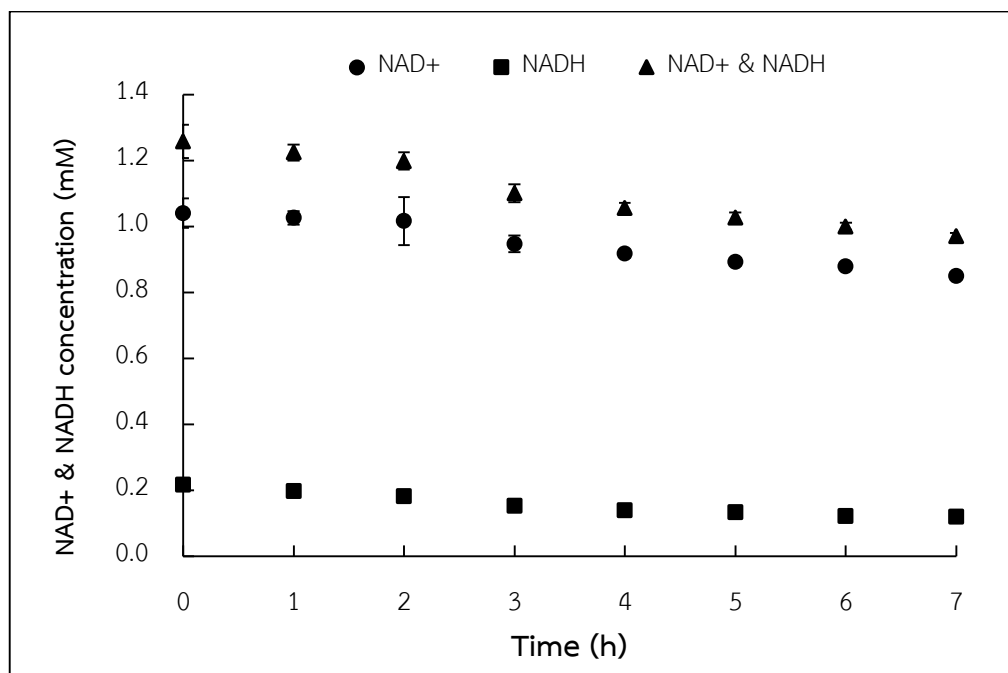
Figure 4.7 Thermal decomposition of NAD$^+$ and NADH at 60 °C.

4.4.2 Conversion of glycerol to lactate

The reaction mixture (4 ml) was composed of 50 mM HEPES-NaOH (pH 7). Metals and enzymes were added to give the final concentrations shown in Table 4.3. The mixture was put in a 10-ml cylindrical vessel and kept at 60°C with stirring. A glycerol solution (160 mM) was continuously supplied to the mixture at a rate of 1 µl/min (0.04 µmol glycerol/ml/min). NAD$^+$ was put in the substrate solution at 4 mM and supplied into the reaction mixture with the substrate to complement the thermal degradation (0.001 µmol NAD$^+$/ml/min). Data represent the averages of triplicate assays.

The result showed that the lactate production rate could be remained almost constant at the expected level (0.04 µmol/min/ml) for the initial 5 h (Figure 4.8). Following this, 11.5 mM lactate could be produced with an overall molar conversion yield of 95.5%. Decrease in the production rate became significant after the initial 5 h, and the conversion yield dropped down to 88% at 7 h. This appeared partly due to the dilution of the reaction mixture (10.5% increase in the total volume at 7 h) caused by the continuous feeding of the substrate solution as well as the loss of enzymes by

the sampling (8.8% decrease in the total concentration at 7 h). Decrease in the production rate might also result from the thermal inactivation of PK_{Tt} (Figure 4.4). Substitution of PK_{Tt} with another pyruvate kinase derived from hyperthermophiles with higher optimum growth temperature than *T. thermophilius* may be a possible means for improving the operational stability of the *in vitro* bioconversion system. The final lactate concentration after the reaction for 7 h was 14.7 mM (The final lactate concentration of theoretical was 16.8 mM for 7 h).

Figure 4.8 Lactate production through the *in vitro* synthetic pathway. Production assays were performed using pure glycerol at 60°C for 7 h. Total concentration of glycerol fed into the reaction mixture was indicated by a dotted line.

4.5 Compatibility of the *in vitro* bioconversion system with crude and model glycerol

The chemical composition of crude glycerol is highly varied with the types of catalysts and feedstocks used for biodiesel production processes [18]. Hansen et al. [55] analyzed the chemical composition of 11 types of crude glycerol obtained from

7 Australian biodiesel manufacturers. They revealed that the glycerol content in the crude glycerol varied in the range of 38% to 96% and up to 16.1% of methanol was contained as an impurity [55]. Moreover, Asad-ur-Rehman et al. [19] reported that a raw glycerol obtained during the biodiesel preparation from sunflower oil contained 50% methanol, which is more abundant than the glycerol content (30%) [19].

In order to assess the compatibility of the *in vitro* system with crude glycerol, a model solution of crude glycerol consisting of 30% (w/v) glycerol and 50% (w/v) methanol was prepared and used as a substrate for lactate production. The model solution was diluted by distilled water to give a final glycerol concentration of 160 mM thereby, the final methanol concentration was 770 mM and supplied into the reaction mixture in the same manner as the lactate production with pure glycerol. Similarly, crude glycerol supplied by Bangchak Petroleum Public Company, Thailand was also diluted by distilled water to give a final glycerol concentration of 160 mM and used as a substrate to replace model crude glycerol.

The result showed that the time profile of the lactate production with the model crude glycerol solution was almost identical to that with pure glycerol. The final lactate concentration after the reaction for 7 h was 14.1 mM. These results were in reasonable agreement with our observation that stabilities of most enzymes involved in the synthetic pathway were not markedly affected by up to 100 mM of methanol. On the other hand, the result of lactate production with the authentic crude glycerol showed that the highest concentration of lactate was 9.2 mM at 7 h. This result was lower than the cases of pure glycerol and model crude glycerol solution. Crude glycerol contains not only methanol but also other impurities such as soap and salt [18]. Therefore, some enzymes in artificial pathway are likely inhibited by some impurities in the authentic crude glycerol [56, 57]. Through the *in vitro* metabolic pathway, glycerol could be converted to lactate with a nearly conversion yield compare with the conventional fermentation (Table 4.4). All results were demonstrated the potential applicability of *in vitro* bioconversion systems with thermophilic enzymes for the conversion of crude glycerol to value-added chemicals.

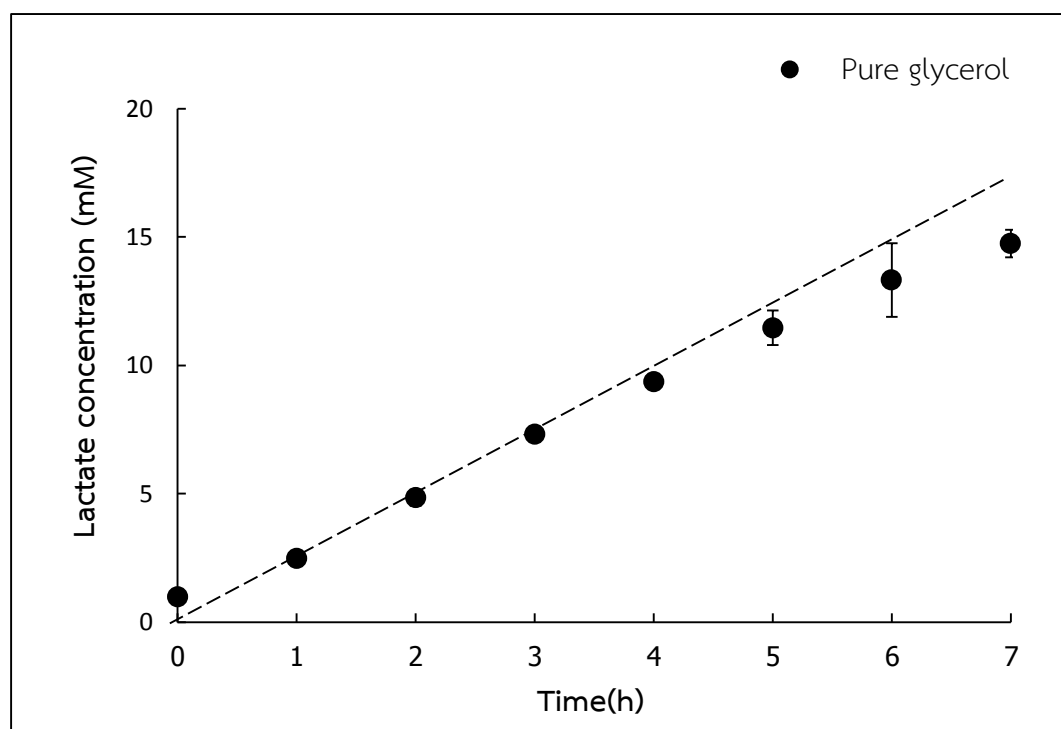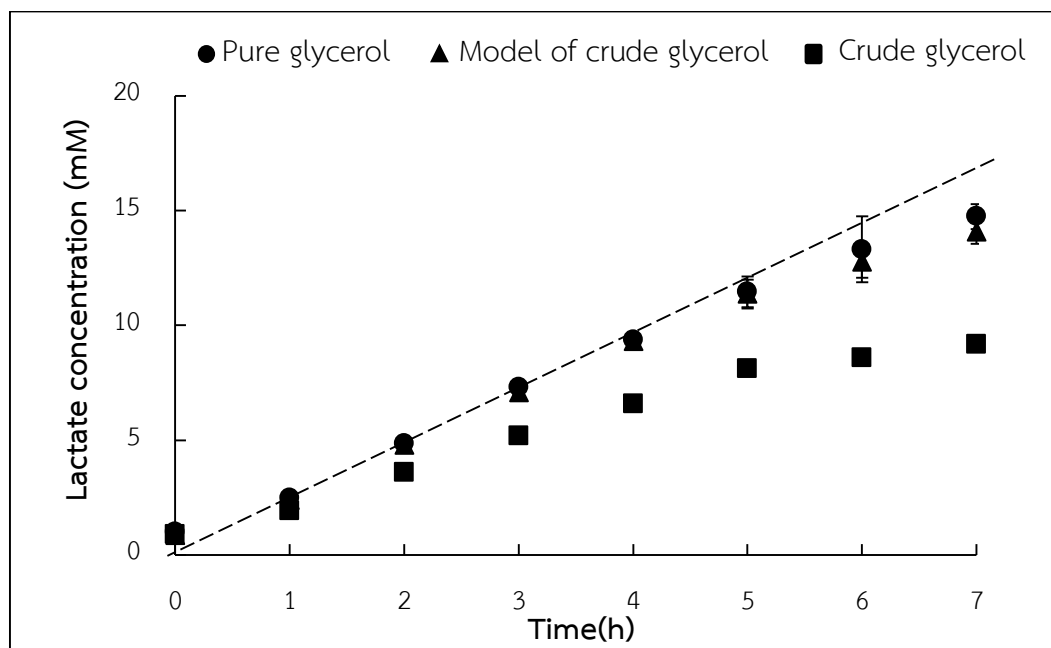**Figure 4.9** Lactate production through the *in vitro* synthetic pathway. Production assays were performed using pure glycerol (circle), model of crude glycerol (triangle) crude glycerol (square) at 60 ℃ for 7 h. Total concentration of glycerol fed into the reaction mixture was indicated by a dotted line.

Table 4.4 Comparison lactate production of an artificial *in vitro* metabolic pathway and conventional fermentation.

Substrate	Yield (%)	Reference
Glycerol	87.8	This study
Model of crude glycerol	83.9	This study
Crude glycerol	54.6	This study
Glycerol	85.0	Mazumdar et al. [28]
Glycerol	78.0	Kangming et al. [58]

CHAPTER V
CONCLUSIONS AND SUGGESTION

In this study, an artificial *in vitro* metabolic pathway for the conversion of glycerol to lactate was constructed with thermostable biocatalytic modules. The *in vitro* pathway consisted of nine thermophilic and hyperthermophilic enzymes (GK, G3PDH, TIM, GAPN, iPGM, ENO, PK, NOX, and LDH) and was designed to balance the intrapathway consumption and regeneration of cofactors. The one-pot conversion of glycerol to lactate through the *in vitro* pathway could be achieved in an almost stoichiometric manner. The final lactate concentration after the reaction for 7 h incubated at 60℃ in 50 mM HEPES-NaOH (pH 7.0) produced by pure glycerol, model crude glycerol production and crude glycerol were 14.7, 14.1 and 9.2 mM respectively. Although the final product concentration obtained in this study was modest, the overall yield (89%) and the production rate of lactate were comparable to those in conventional fermentation processes [59]. Besides, the *in vitro* bioconversion system can be operated in a simple buffer solution and thus would markedly simplify down-stream processes including product recovery and purification. Furthermore, the *in vitro* bioconversion system exerted almost identical performance in the presence of methanol, demonstrating the potential of thermophilic enzyme based *in vitro* metabolic engineering approaches in the utilization of crude glycerol as a starting material for the production of value-added chemicals. On the other hand, although their contents are generally less abundant than those of methanol, crude glycerol contains many other impurities, including fatty acids, soap, and salts which, likely inhibit the activities some enzymes.

The chemical composition of crude glycerol mainly varies with the type of catalyst used to produce biodiesel, the transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock and whether the methanol and catalysts were recovered. The variety of crude glycerol from many company would be need in future study. To assess their compatibility with *in vitro* systems. Moreover, the enzymes required for the construction of an *in vitro* artificial metabolic pathway have

to separately prepared in different batchs, leading to the complicate perations in large scale. The genes should be assembled in an artificial operon and co-expressed in a single recombinant *E. coli* [12].

REFERENCES

[1]   Rabinovitch-Deere, C.A., Oliver, J.W., Rodriguez, G.M., and Atsumi, S. Synthetic biology and metabolic engineering approaches to produce biofuels. Chemical Review 113(7) (2013): 4611-32.

[2]   Krutsakorn, B., et al. In vitro production of n-butanol from glucose. Metabolic Engineering 20 (2013): 84-91.

[3]   Bond-Watts BB1, B.R., Chang MC. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Natural Chemical Biology 7(4) (2011): 222-7.

[4]   Ye, X., Honda, K., Sakai, S., Okano, K., Omasa, T., Hirota, R., Kuroda, A., and Ohtake, H. . Synthetic Metabolic engineering-a novel, simple technology for designing a chimeric metabolic pathway. Microbial Cell Factories 11 (2012): 120-130.

[5]   Jan-Karl, G., et al. Cell-Free Metabolic Engineering: Production of Chemicals by Minimized Reaction Cascades. Chemisty & Sustainability 5(11) (2012): 2165-72.

[6]   Ye, X., Honda, K., Morimoto, Y., Okano, K., and Ohtake, H. Direct conversion of glucose to malate by synthetic metabolic engineering. Biotechnology 164(1) (2013): 34-40.

[7]   You, C., et al. Enzymatic transformation of nonfood biomass to starch. Proceeding of National Academy of Science USA 110(18) (2013): 7182-7.

[8]   Woodward, J., Orr, M., Cordray, K., and Greenbaum, E. Biotechnology: Enzymatic production of biohydrogen. Nature 405(6790) (2000): 1014-15.

[9]   Zhang, Y.H.P., Evans, B.R., Mielenz, J.R., Hopkins, R.C., and Adams, M.W.W. High-Yield Hydrogen Production from Starch and Water by a Synthetic Enzymatic Pathway. PLoS ONE 2(5) (2007): e456.

[10]   Opgenorth, P.H., Korman, T.P., and Bowie, J.U. A synthetic biochemistry molecular purge valve module that maintains redox balance. Nature Communication 5 (2014): 4113-20.

[11]   Zhu, Z., Kin Tam, T., Sun, F., You, C., and Percival Zhang, Y.H. A high-energy-density sugar biobattery based on a synthetic enzymatic pathway. <u>Nature Communication</u> 5 (2014): 3026-33.

[12]   Ninh, P.H., Honda, K., Sakai, T., Okano, K., and Ohtake, H. Assembly and multiple gene expression of thermophilic enzymes in *Escherichia coli* for *in vitro* metabolic engineering. <u>Biotechnology and Bioengineering</u> 112(1) (2015): 189-196.

[13]   Owusu, R.K. and Cowan, D.A. Correlation between microbial protein thermostability and resistance to denaturation in aqueous: organic solvent two-phase systems. <u>Enzyme and Microbial Technology</u> 11(9) (1989): 568-74.

[14]   Atomi, H. Recent progress towards the application of hyperthermophiles and their enzymes. <u>Current Opinion in Chemical Biology</u> 9(2) (2005): 166-73.

[15]   Pennacchio, A., Pucci, B., Secundo, F., La Cara, F., Rossi, M., and Raia, C.A. Purification and characterization of a novel recombinant highly enantioselective short-chain NAD(H)-dependent alcohol dehydrogenase from *Thermus thermophilus*. <u>Applied Environmental Microbiology</u> 74(13) (2008): 3949-58.

[16]   Nguyen, A.D.Q., Kim, Y.G., Kim, S.B., and Kim, C.-J. Improved tolerance of recombinant *Escherichia coli* to the toxicity of crude glycerol by overexpressing trehalose biosynthetic genes (otsBA) for the production of β-carotene. <u>Bioresource Technology</u> 143(0) (2013): 531-7.

[17]   Venkataramanan, K., Boatman, J., Kurniawan, Y., Taconi, K., Bothun, G., and Scholz, C. Impact of impurities in biodiesel-derived crude glycerol on the fermentation by *Clostridium pasteurianum* ATCC 6013. <u>Applied Microbiology and Biotechnology</u> 93(3) (2012): 1325-35.

[18]   Yang, F., Hanna, M.A., and Sun, R. Value-added uses for crude glycerol--a byproduct of biodiesel production. <u>Biotechnology Biofuels</u> 5 (2012): 13.

[19]   Asad-ur-Rehman, Saman Wijesekara R.G, Nomura N., Sato S., and Matsumura, M. Pre-treatment and utilization of raw glycerol from sunflower oil biodiesel for growth and 1,3-propanediol production by *Clostridium butyricum*. <u>Chemical Technology and Biotechnology</u> 83(7) (2008): 1072-80.

[20]   Xu, K. and Xu, P. Efficient calcium lactate production by fermentation coupled with crystallization-based in situ product removal. <u>Bioresource Technology</u> 163(0) (2014): 33-9.

[21]   Zhou, J., Zhang, H., Meng, H., Zhang, Y., and Li, Y. Production of optically pure d-lactate from CO2 by blocking the PHB and acetate pathways and expressing d-lactate dehydrogenase in cyanobacterium *Synechocystis* sp. PCC 6803. <u>Process Biochemistry</u> 49(12) (2014): 2071-7.

[22]   Upadhyaya, B.P., DeVeaux, L.C., and Christopher, L.P. Metabolic engineering as a tool for enhanced lactic acid production. <u>Trends Biotechnology</u> 32(12) (2014): 637-44.

[23]   Wee, Y.J., Yun, J.S., Kim, D., and Ryu, H.W. Batch and repeated batch production of L (+)-lactic acid by *Enterococcus faecalis* RKY1 using wood hydrolyzate and corn steep liquor. <u>Industrial Microbiology Biotechnology</u> 33(6) (2006): 431-5.

[24]   Abdel-Rahman, M.A., Tashiro, Y., and Sonomoto, K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. <u>Biotechnology</u> 156(4) (2010): 286-301.

[25]   Dien B.S., Nichols N.N., and Bothast, R.B. Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars. <u>Industrial Microbiology & Biotechnology</u> 27 (2001): 259-64.

[26]   Zhu, Y., Eiteman, M.A., DeWitt, K., and Altman, E. Homolactate fermentation by metabolically engineered *Escherichia coli* strains. <u>Applied Environmental Microbiology</u> 73(2) (2007): 456-64.

[27]   Zhao, B., Wang, L., Ma, C., Yang, C., Xu, P., and Ma, Y. Repeated open fermentative production of optically pure l-lactic acid using a thermophilic *Bacillus* sp. strain. <u>Bioresource Technology</u> 101(16) (2010): 6494-8.

[28]   Mazumdar, S., Clomburg, J.M., and Gonzalez, R. *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. <u>Applied Environmetal Microbiology</u> 76(13) (2010): 4327-36.

[29]   Zhang, Y.H., Sun, J., and Zhong, J.J. Biofuel production by *in vitro* synthetic enzymatic pathway biotransformation. <u>Current Opinion Biotechnology</u> 21(5) (2010): 663-9.

[30]   Zhang, Y.H. Production of biofuels and biochemicals by *in vitro* synthetic biosystems: Opportunities and challenges. Biotechnology Advances (2014): 1-7.

[31]   Scopes, R.K. Glycolysis in cell-free systems New Beer in an Old Bottle: Eduard Buchner and the Growth of Biochemical Knowledge (1997): 151-8.

[32]   Buchner, E. Alkoholische Gärung ohne Hefezellen. Berichte der deutschen chemischen Gesellschaft 30(1) (1897): 1110-3.

[33]   Patrick C. Hallenbeck, J.R.B. Biological hydrogen production; fundamentals and limiting processes. International Journal of Hydrogen Energy 27 (2002): 1185-1193.

[34]   DebabrataDas, T.N.V. Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26 (2001): 13-28.

[35]   Myung, S., et al. *In vitro* metabolic engineering of hydrogen production at theoretical yield from sucrose. Metabolic Engineering 24 (2014): 70-7.

[36]   Wikler, A., Arch. Gen. Enzymatic production of biohydrogen. Nature 405 (2000): 1014-5.

[37]   Atanassovb, S.C.B.a.P. Enzymatic biofuel cells for implantable and micro-scale devices. Fuel Chemistry and Technology 49(2) (2004): 476-7.

[38]   Zhu, Z., Sun, F., Zhang, X., and Zhang, Y.H. Deep oxidation of glucose in enzymatic fuel cells through a synthetic enzymatic pathway containing a cascade of two thermostable dehydrogenases. Biosens Bioelectron 36(1) (2012): 110-5.

[39]   Xu, S. and Minteer, S.D. Enzymatic Biofuel Cell for Oxidation of Glucose to CO_2. ACS Catalysis 2(1) (2012): 91-94.

[40]   Satoh Y., Tajiima K., Tannai H., and Munekata M. Enzyme-Catalyzed Poly(3-Hydroxybutyrate) Synthesis from Acetate with CoA Recycling and NADPH Regeneration *in vitro* Bioscinec and Bioengineering 95(4) (2002): 335-41.

[41]   Li, H., Cann, A.F., and Liao, J.C. Biofuels: biomolecular engineering fundamentals and advances. Annual Review Chemical Biomolecular Engineering 1 (2010): 19-36.

[42]   Zhang, Y.H.P. Simpler Is Better: High-Yield and Potential Low-Cost Biofuels Production through Cell-Free Synthetic Pathway Biotransformation (SyPaB). ACS Catalysis 1(9) (2011): 998-1009.

[43]   Bornscheuer, U.T., Huisman, G.W., Kazlauskas, R.J., Lutz, S., Moore, J.C., and Robins, K. Engineering the third wave of biocatalysis. Nature 485(7397) (2012): 185-94.

[44]   Fjeld, C.C., Birdsong, W.T., and Goodman, R.H. Differential binding of NAD$^+$ and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor. Proceeding of the National Academy of Science USA 100(16) (2003): 9202-7.

[45]   Tan, A.S. and Berridage, M.V. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. Immunological Methods (238) (2000): 59-68.

[46]   Yin, L.M., Wei, Y., Wang, Y., Xu, Y.D., and Yang, Y.Q. Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells. Internation Joural Medical Science 10(1) (2013): 68-72.

[47]   Samul, D., Leja, K., and Grajek, W. Impurities of crude glycerol and their effect on metabolite production. Annals Microbiology 64 (2014): 891-8.

[48]   Almeida, J.R.M., Favaro, L.C.L., and Quirino, B.F. Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol Biofuels (5) (2012): 48-63.

[49]   Ruhal, R., Aggarwal, S., and Choudhury, B. Suitability of crude glycerol obtained from biodiesel waste for the production of trehalose and propionic acid. Green Chemistry 13(12) (2011): 3492-98.

[50]   Hu, S., Luo, X., Wan, C., and Li, Y. Characterization of crude glycerol from biodiesel plants. Agriculture and Food Chemistry 60(23) (2012): 5915-21.

[51]   Koga Y., Haruki, M., Morikawa M., and Kanaya, S. Stabilities of Chimeras of Hyperthermophilic and Mesophilic Glycerol Kinases Constructed by DNA Shuffling Bioscience and Bioengineering 91(6) (2001): 551-6.

[52]   Yokoyama S., Hirota, H., Kigawa, T., Yabuki, T., Shirouzu, M., Terada, T., Ito, Y., Matsuo, Y., Kuroda, Y., Nishimura, Y., Kyogoku, Y., Miki, K., Kuramitsu, R.M.S.. Structural genomics projects in Japan. Nature structural biology 7 (2000): 943-5.

[53]   Jia, B., et al. Hexameric ring structure of a thermophilic archaeon NADH oxidase that produces predominantly H_2O. FEBS J 275(21) (2008): 5355-66.

[54]   Matsubara, K., Yokooji, Y., Atomi, H., and Imanaka, T. Biochemical and genetic characterization of the three metabolic routes in *Thermococcus kodakarensis* linking glyceraldehyde 3-phosphate and 3-phosphoglycerate. Molecular Microbiology 81(5) (2011): 1300-12.

[55]   C. F. Hansen , A.H., B. P. Mullan, K. Moore, M. Trezona-Murray, R. H. King and J. R. Pluske. A chemical analysis of samples of crude glycerol from the production of biodiesel in Australia, and the effects of feeding crude glycerol to growing-finishing pigs on performance, plasma metabolites and meat quality at slaughter. Animal Production Science 49 (2009): 154-161.

[56]   Zhao, H. Effect of ions and other compatible solutes on enzyme activity, and its implication for biocatalysis using ionic liquids. Journal of Molecular Catalysis B: Enzymatic 37(1-6) (2005): 16-25.

[57]   Duddridge, J.E. and Wainwright, M. Effect of sodium chloride on enzyme activity and synthesis in river sediments. Environmental Technology Letters 1(7) (2008): 319-326.

[58]   Kangming, T. High-efficiency conversion of glycerol to D-lactic acid with metabolically engineered *Escherichia coli*. African Journal of Biotechnology 11(21) (2012).

[59]   Okano, K., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Applied Microbiology and Biotechnology 85(3) (2010): 413-23.

APPENDICES

APPENDIX **1**

LB medium

Composition per 1 liter

10 g	Bacto tryptone
5 g	Yeast extract
10 g	NaCl

Dissolve all compositions with 800 ml deionized water, adjust the pH to 7.0 with 6 M NaOH. Adjust volume of solution to 1 liter with deionized water. Autoclave at 121 °C, 15 lb/in2 for 15 min. For medium containing agar add bactoagar 15 g per liter.

APPENDIX 2

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

30% Acrylamide, 0.8% bis acrylamide, 100 ml

Acrylamide 29.2 g

N, N´ methylene bis acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane 18.2 g

Adjust pH to 8.8 and adjust volume to 100 ml with distilled water.

2 M Tris Cl pH 8.8

Tris (hydroxymethyl) aminomethane 24.2 g

Adjust pH to 8.8 and adjust volume to 100 ml with distilled water.

0.5 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane 6.1 g

Adjust pH to 6.8 and adjust volume to 100 ml with distilled water.

1 M Tris Cl pH 6.8

Tris (hydroxymethyl) aminomethane 12.1 g

Adjust pH to 6.8 and adjust volume to 100 ml with distilled water.

Solution B (SDS-PAGE)

2 M Tris Cl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS-PAGE)

2 M Tris Cl pH 6.8 50 ml

10% SDS 4 ml

Distilled water 46 ml

2. SDS-PAGE

10% separating gel

30% acrylamide solution 3.33 ml

Solution B 2.5 ml

Distilledl water 5.0 ml

10% Ammonium sulfate 50 µl

TEMED 10 µl

5% stacking gel

30% acrylamide solution 0.67 ml

Solution B 1.0 ml

Distilled water 2.3 ml

10% Ammonium sulfate 30 µl

TEMED 5.0 µl

Sample buffer

1M Tris Cl pH 6.8 0.6 ml

50% glycerol 5.0 ml

10% SDS 2.0 ml

2 mercaptoethanol 0.5 ml

1% bromphenol blue 1.0 ml

Distilled water 0.9 ml

4x of sample buffer is mixture sample to 1x. The mixture heated 5 min in
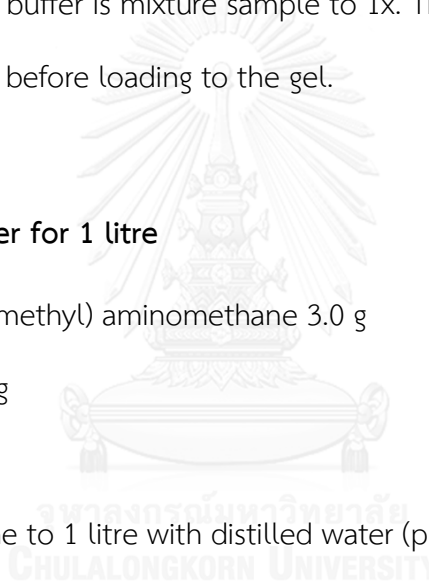
boiling water before loading to the gel.

Electrophoresis buffer for 1 litre

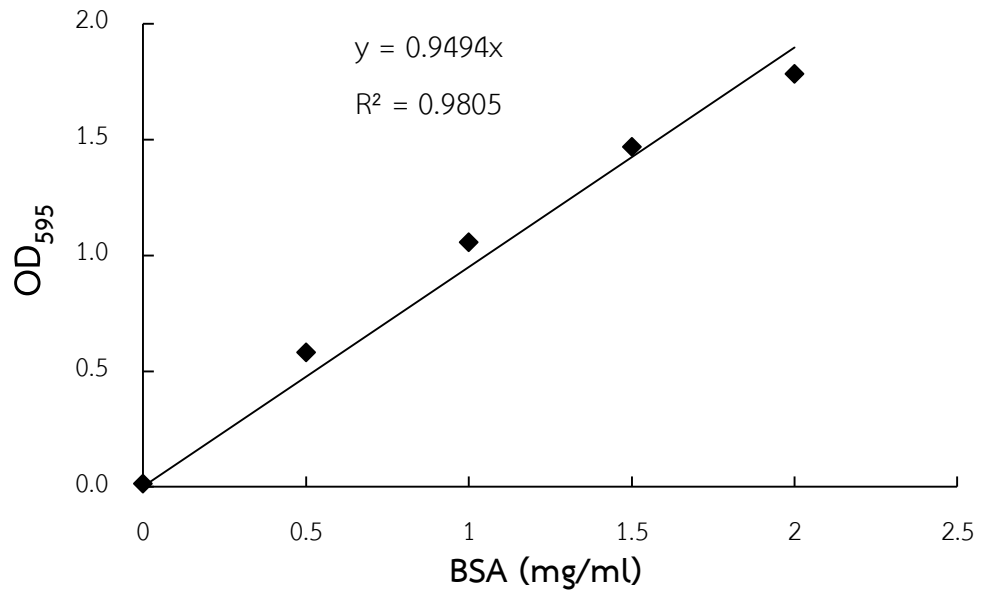Tris (hydroxymethyl) aminomethane 3.0 g

Glycine 14.4 g

SDS 1.0 g

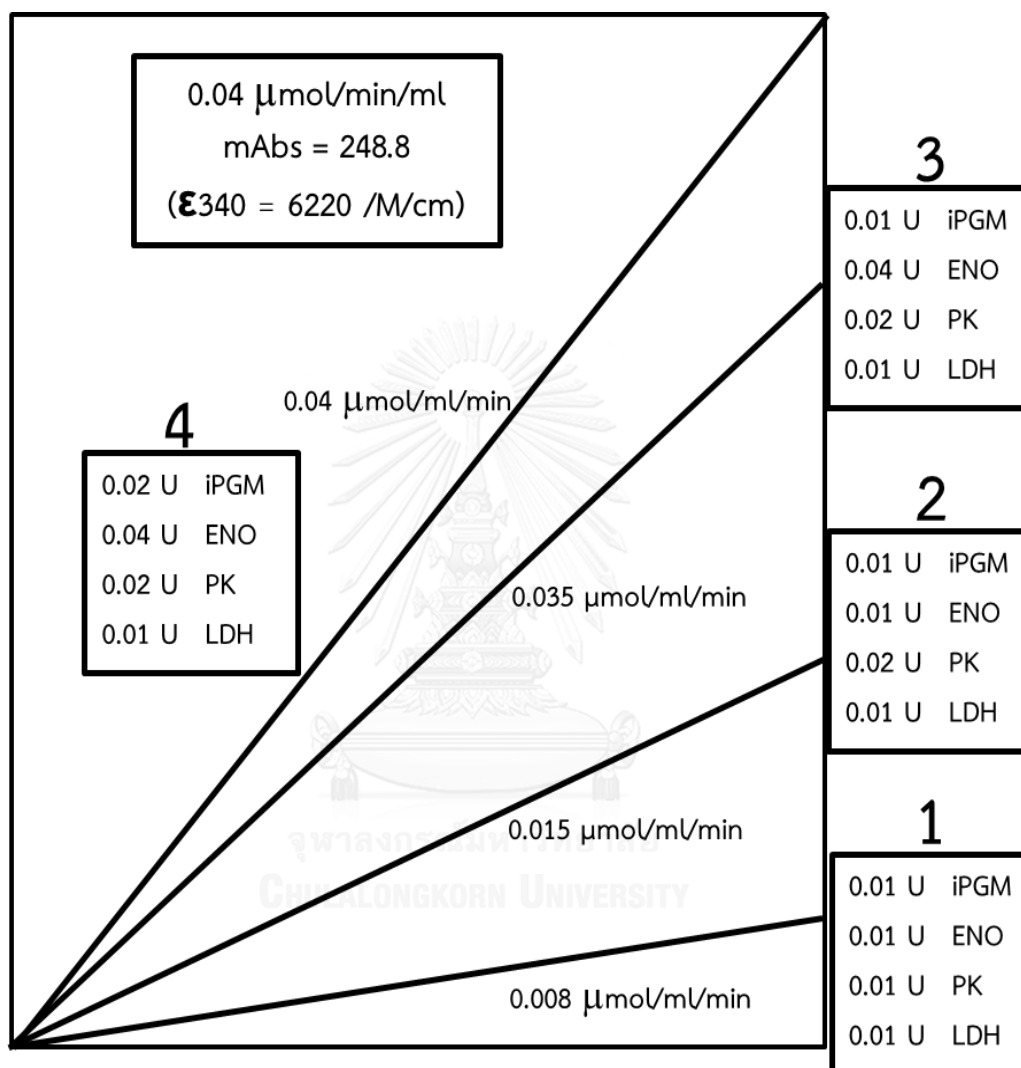Adjust volume to 1 litre with distilled water (pH 8.3).

APPENDIX 3

Standard curve for protein determination

$y = 0.9494x$

$R^2 = 0.9805$

OD$_{595}$ vs BSA (mg/ml)

APPENDIX 4

The real-time monitoring of the flux through the *in vitro* pathway by

monitoring the concomitant consumption or production of NAD(P)H at 340 nm.

For example of third part of artificial pathway from 3-PG to lactate. The real-time monitoring technique enables us to identify rate-limiting enzymes in an *in vitro* pathway by increasing the concentration of each enzyme, one another was shown in number 1, 2, 3 and 4 respectively.

VITA

Miss Chalisa Jaturapaktrarak was born on Aug 5, 1989 in Bangkok, Thailand. She graduated with Bachelor's degree from Department of Microbiology, Faculty of Science, Srinakharinwirot University in 2012. Then, she continue her study as a graduate student in the Department of Microbiology at Chulalongkorn University and some part of this work published in Jaturapaktrarak, C., Napathorn, S. C., Cheng, M., Okano, K., Ohtake, H. and Honda, K. In vitro conversion of glycerol to lactate with thermophilic enzymes Bioresources and Bioprocessing 1 (2014): 18-25 and the 26th annual meeting of the Thai society for biotechnology and international conference in 2014. She finished her postgraduate study with Master degree of Science 2014. Her present address is 79/184 Moo#2 , Lapawan 9 village, Liab-Klong Road, Bangplub, Pak-Kret, Nonthaburi, Thailand, 11120, Contact number is 091-716-8325 and e-mail: melody_beerody@hotmail.com.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY