การเพิ่มผลผลิตกรดแอล(+)-แลกติกใน *Rhizopus oryzae* NRRL395 โดยการยับยั้งเมแทบอลิซึม
ในการสร้างเอทานอล

นางสาวสิตานัน ธิติประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

ENHANCEMENT OF L(+)-LACTIC ACID PRODUCTION IN *Rhizopus oryzae* NRRL395 BY INHIBITION OF ETHANOL METABOLIC PATHWAY

Miss Sitanan Thitiprasert

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2013

Thesis Title	ENHANCEMENT OF L(+)-LACTIC ACID PRODUCTION IN Rhizopus oryzae NRRL395 BY INHIBITION OF ETHANOL METABOLIC PATHWAY
By	Miss Sitanan Thitiprasert
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Nuttha Thongchul, Ph.D.
Thesis Co-Advisor	Sarintip Sooksai, Ph.D.
	Kentaro Kodama, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Polkit Sangvanich, Ph.D.)

.....Thesis Advisor
(Assistant Professor Nuttha Thongchul, Ph.D.)

.....Thesis Co-Advisor
(Sarintip Sooksai, Ph.D.)

.....Thesis Co-Advisor
(Kentaro Kodama, Ph.D.)

.....Examiner
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

.....Examiner
(Kittinan Komolpis, Ph.D.)

.....External Examiner
(Assistant Professor Suwattana Pruksasri, Ph.D.)

สิตานัน ธิติประเสริฐ : การเพิ่มผลผลิตกรดแอล(+)-แลกติกใน *Rhizopus oryzae* NRRL395 โดยการยับยั้งเมแทบอลิซึมในการสร้างเอทานอล. (ENHANCEMENT OF L(+)-LACTIC ACID PRODUCTION IN *Rhizopus oryzae* NRRL395 BY INHIBITION OF ETHANOL METABOLIC PATHWAY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. ศรินทิพ สุกใส, ดร. Kentaro Kodama, 112 หน้า.

ในการศึกษาการควบคุมวิถีเมแทบอลึซึมของการผลิตเอทานอลในการตรึงเซลล์ *Rhizopus oryzae* NRRL395 เพื่อการเพิ่มผลผลิตกรดแอล-แลกติก นั้นศึกษาโดยการใช้สารควบคุมเอนไซม์ไพรูเวทดีคาร์บอกซีเลสและแอลกอฮอล์ดีไฮโดรจีเนส ซึ่งสารควบคุมได้ถูกคัดเลือกมาจากงานวิจัยที่เกี่ยวข้อง ซึ่งสารควบคุมชนิดต่างๆจะถูกศึกษาเปรียบเทียบอิทธิพลของการเติมในอาหารเลี้ยงเชื้อที่มีชนิดที่แตกต่างกันต่อการผลิตผลิตภัณฑ์และการทำงานของเอนไซม์ ซึ่งจากการศึกษาการควบคุมการทำงานของเอนไซม์แอลกอฮอล์ดีไฮโดรจีเนสในระดับขวดเขย่า พบว่า 1,2-diazole และ 2,2,2-trifluoroethanol คือสารควบคุมที่มีประสิทธิภาพที่ส่งผลควบคุมต่อการทำงานของเอนไซม์แอลกอฮอล์ดีไฮโดรจีเนส อย่างไรก็ตาม การใช้สารทั้ง 2 ชนิดไม่เพียงพอต่อการควบคุมการผลิตเอทานอล ซึ่งเอทานอลยังคงถูกผลิตอยู่ในเชื้อราสายพันธุ์นี้ ดังนั้นจึงทำการศึกษาการควบคุมการทำงานของเอนไซม์ไพรูเวทดีคาร์บอกซีเลสซึ่งเป็นเอนไซม์อีกชนิดที่เกี่ยวข้องในการผลิตเอทานอล ซึ่งพบว่า 4-methylpyrazole ความเข้มข้น 0.1 มิลลิโมลาร์ และ β-hydroxypyruvate ความเข้มข้น 1 ไมโครโมลาร์ ส่งผลต่อการเพิ่มการผลิตกรดแอล-แลกติกอีกทั้งลดการผลิตเอทานอลใน *R. oryzae* จากนั้นสารควบคุมเอนไซม์ทั้ง 4 ชนิดดังกล่าว ได้ถูกนำมาศึกษาต่อในระดับถังหมักแบบเบดสถิต โดยทำการแปรความเข้มข้นของค่าการละลายออกซิเจน พบว่า การเติม 2,2,2-trifluoroethanol ลงในอาหารเลี้ยงเชื้อและทำการควบคุมค่าการละลายออกซิเจนที่ 80 เปอร์เซ็นต์ นั้นส่งผลต่อการเพิ่มการผลิตกรดแอล-แลกติกได้มากที่สุดอีกทั้งยังช่วยลดการผลิตเอทานอล ซึ่งเมื่อทำการเปรียบเทียบกับการหมัก *R. oryzae* โดยที่ไม่มีการเติมสารควบคุมลงในอาหารเลี้ยงเชื้อ พบว่าสารดังกล่าวเพิ่มการผลิตกรดแอล-แลกติกขึ้น 24 เปอร์เซ็นต์ นอกจากนี้ในการศึกษาอิทธิพลของสารควบคุมดังกล่าวต่อกิจกรรมจำเพาะของเอนไซม์ที่เกี่ยวข้องในวิถีเมทาบอลิซึมของ *R. oryzae* NRRL395 นั้น การเติมสารควบคุมลงในอาหารเลี้ยงเชื้อโดยตรงนั้นไม่เพียงแต่จะส่งผลควบคุมเอนไซม์เป้าหมาย แต่สารควบคุมนั้นยังส่งผลต่อวิถีเมแทบอลึซึมที่เกี่ยวข้องกันอีกด้วย

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5072509423 : MAJOR BIOTECHNOLOGY
KEYWORDS: *RHIZOPUS ORYZAE* / LACTIC ACID / LACTATE DEHYDROGENASE / ALCOHOL DEHYDROGENASE / PYRUVATE DECARBOXYLASE / REGULATION / IN VIVO

SITANAN THITIPRASERT: ENHANCEMENT OF L(+)-LACTIC ACID PRODUCTION IN *RHIZOPUS ORYZAE* NRRL395 BY INHIBITION OF ETHANOL METABOLIC PATHWAY. ADVISOR: ASST. PROF. NUTTHA THONGCHUL, Ph.D., CO-ADVISOR: SARINTIP SOOKSAI, Ph.D., KENTARO KODAMA, Ph.D., 112 pp.

The regulation of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the living culture of *Rhizopus oryzae* was studied. The in vitro enzyme inhibitors were selected from the literatures. They were added into the fermentation of immobilized *R. oryzae* and their concentration effects on growth, metabolite production, and changes in the specific enzyme activities were investigated during the shake flask culture. It was found that 1,2-diazole and 2,2,2-trifluoroethanol were found to be the effective ADH regulators. Nevertheless, regulating only ADH could not fully limit ethanol during fermentation by *R. oryzae*. PDC was further suppressed by various regulators. It was found that among the regulators studied, 0.1 mM 4-methylpyrazole and 1.0 μM β-hydroxypyruvate successfully provided the enhancement of lactic acid production as well as the decreasing ethanol formation. Later on, all of these potential 4 regulators were tested with dissolved oxygen for their combinatorial effects on growth, metabolite production, and the specific enzyme activities in the static bed bioreactor. The results revealed that the effect of enzyme regulators was more profound at the higher DO level. It was found that DO level of 80% in the fermentation medium containing 2,2,2-trifluoroethanol exhibited the highest lactic acid production as well as reducing ethanol production. This was about 24% increasing yield when compared with that of the control fermentation. However, the results shown in this study implied that the regulation of ethanol fermentative pathway by adding the regulators during fermentation did not only affect the targeted enzymes but it also caused the dynamic change in the conversion of all metabolites in the living *R. oryzae* in order to maintain the balanced flux for cellular growth and maintenance.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2013 Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This research paper is in partial fulfillment of the requirements for the Degree of Doctor of Philosophy of Science (Biotechnology) from Chulalongkorn University. This work has been fully supported by Thailand Research Fund and the Graduate School of Chulalongkorn University via the TRF Master Research Grant (MRG-WII51E053) and partially funded by the National Research University Project of Commission on Higher Education (CHE) and the Ratchadapiseksomphot Endowment Fund (AM1026A).

I deeply impress my thesis advisor, Asst. Prof. Dr. Nuttha Thongchul, who gave me an inspiration for working and solving the problems. This research could not be successful without her kind assistance, guidance, and encouragement throughout this research. Furthermore, I also thank you my thesis co-advisors, Dr. Sarintip Sooksai and Dr. Kentaro kodama, for their advice, comments, kindness, and support. I wish to thank Assoc. Prof. Dr. Amorn Petchsom, Assoc. Prof. Dr. Polkrit Sangvanich, Assoc. Prof. Dr. Nattaya Ngamrojavanich, Dr. Kittinan Komolpis, and Asst. Prof. Dr. Suwattana Pruksasri for sacrificing their time to be committees and giving recommendation.

My thanks are extended to OeAD scholarship which gave me the opportunity for achieving the technology grants Southeast Asia. This opportunity provided me a new experience and wide perspective.

Special thanks to my NT group members at the Institute of Biotechnology and Genetic Engineering for their support and valuable friendship.

I wish to say thank you very much to my beloved family, especially my parents, who always give me suggestions and help me with everything.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVATION

mL	Milliliter
L	Liter
µL	Microliter
µmole	Micromole
µM	Micromolar
M	Molar
µm	Micrometer
U/mg protein	Unit enzyme per microgram protein
g	Gram
g/L	Gram per liter
g/g	Gram product per gram substrate
g/L·h	Gram per liter per hour
g/mol	Gram per mole
min	Minute
h	Hour
rpm	Round per minute
vvm	Air volume per volume of medium per minute
nm	Nanometer
psi	Pounds per square inch
°C	Degree Celsius
LDH	Lactate dehydrogenase
ADH(f)	Alcohol dehydrogenase (Ethanol reduction)
ADH(b)	Alcohol dehydrogenase (Ethanol oxidation)
PDC	Pyruvate decarboxylase

CHAPTER I

INTRODUCTION

1.1 Background

Lactic acid has long been widely used in food, pharmaceutical, cosmetic, and chemical industries. Lactic acid production is currently attracting a great deal of research and development. A review published in 1995 stated that 85% of lactic acid in the USA was used in food and food-related applications. The rest was used in an emerging application for the production of polylactic acid a renewable, biodegradable, and biocompatible polymer, which provided an environmental friendly alternative to biodegradable plastics derived from petrochemical materials (Datta et al., 1995; Lunt, 1998; and Södergård and Stolt, 2002). With the rapid technology development and commercialization of this biopolymer, its application has increased considerably. This resulted in an approximately 20–30% of the 120,000 ton global production of lactic acid was estimated to be used in these new applications in 2005 (Datta et al., 2006).

Lactic acid naturally exists in two optical isomers: D(–)-lactic acid and L(+)-lactic acid. Since elevated levels of the D-isomer are harmful to human, L(+)-lactic acid is the preferred isomer for food-related and pharmaceutical industries (Tay and Yang, 2002). Lactic acid can be produced commercially by either chemical synthesis or fermentation. The chemical synthesis results in a racemic mixture of the two isomers, while the fermentation process can yield an optically pure form of lactic acid or racemate, depending on microorganisms, substrates and fermentation conditions employed in the production process. The most common chemical synthesis used in lactic acid production is the hydrolysis of lactonitrile derived from acetaldehyde and hydrogen cyanide, the feedstocks from crude oil (Zhang et al., 2007 and Narayanan, 2004). Besides using nonrenewable feedstocks, chemical synthesis requires high elevated temperature and pressure; thus it is considered as a non-environmental friendly process. On the other hand, microorganisms can consume renewable raw materials including molasses, starch (cornstarch, wheat starch, and potato starch), and lignocelluloses (corn cobs and wood materials) as a substrate in lactic acid fermentation (Bullet, 2004). Currently lactic acid is commercially produced via bacterial fermentation (Litchfield, 1996). Lactic acid bacteria (LAB) have received a wide interest because they provide high growth rate

and product yield. LAB usually produce a racemic mixture of L(+)- and D(-)-lactic acids. For polylactic acid synthesis; however, an optically pure isomer of lactic acid is required (Tsai and Moon, 1998). Therefore, after LAB fermentation, costly purification of stereoisomer of lactic acid is necessary. Also, bacterial fermentation is usually carried out in a rich (complex) medium at a relatively high pH (>5), whereby lactic acid is produced as lactate salt. Homolactic acid bacteria, especially some Lactobacilli, are extremely fastidious and require one or more amino acids for growth. Rich nutrient supplements required in homolactic acid bacterial fermentation not only increase the raw material cost, but also make the final product recovery and purification complicated. The expensive downstream process contributes to the increase in the overall production cost of lactic acid. Using low cost substrates such as starch and pentoses directly in fermentation can help reduce the production cost. However, homolactic acid bacteria cannot efficiently ferment starch and pentoses. As previously discussed, homolactic acid bacterial fermentation is likely limited by an inability to ferment starch and pentoses, a complex nutrient requirement, a relatively high pH requirement, and its production of a racemic mixture of lactic acid (Tay and Yang, 2002).

Recently, there has been increasing interest in fungal fermentation to produce an optically pure L-lactic acid (Hang and Suntornsuk, 1994; Soccol et al., 1994a,b; Dong et al., 1996; Kosakai et al., 1997; Lin et al., 1998; Park et al., 1998; Sun et al., 1999; Woiciechowski et al., 1999; Yin et al., 1998; and Zhou et al., 1999). *Rhizopus oryzae* has been recognized as a suitable candidate for lactic acid production. The production of L-lactic acid using a surface culture of *Rhizopus* was reported in 1911 (Ehrlich, 1911). An efficient submerged fermentation using fungal species for the production of L-lactic acid was first reported in 1936 (Lockwood et al., 1936). However, an increased research interest has been given to lactic acid fermentation by fungal species in recent decades. *Rhizopus* strains grow better under the nitrogen limited environment than the lactic acid bacteria (Yu and Hang, 1989; Soccol et al., 1994a,b; and Rosenberg and Kristofikova, 1995). When starch-based material is used as a substrate, only small amounts of inorganic salts and inorganic nitrogen are required for lactic acid production using Rhizopus fungi. Separation of the fungal biomass from fermentation broth is easy because the filamentous or pellet form leads to a simple and inexpensive downstream process. In addition, as a by-product from lactic acid production, fungal biomass from *Rhizopus* strains can be used in biosorption processes for purification of contaminated effluents (Tobin et al., 1984 and Pochanavanich and Suntornsuk, 2002), fungal chitosan production

(Pochanavanich and Suntornsuk, 2002 and Yoshihara et al., 2003), and as additive in animal feeds to improve the feed quality (Kusumaningtyas, 2006).

However, lactic acid fermentation by *R. oryzae* is an aerobic process, requiring vigorous aeration, and usually has a lower productivity (~ 2 g/L·h) and product yield (85% vs. 95% for homolactic acid fermentation). The lower product yield from fungal fermentation is partially due to the formation of other by-products, including ethanol and fumaric acid. The lower productivity can be attributed to the low reaction rate caused by mass transfer limitation inside the fungal mycelia clumps or pellets (Park et al., 1998). Fermentation with filamentous fungi is also complicated by the increased broth viscosity to mycelia growth and reduced oxygen transfer, which is critical to lactic acid production. Under oxygen-limited conditions, more ethanol and less lactic acid are produced due to the increased activity of alcohol dehydrogenase (Skory et al., 1998).

The highly branched fungal mycelia cause complex (viscous) broth rheology and difficulty in mixing and aeration in conventional agitated-tank fermentor (Tay and Yang, 2002). To avoid the problem in mixing, fungal fermentation by *R. oryzae* can be carried out using the substrate (starch) present in a solid state (such as in rice, wheat, and corn grain), but lactic acid yield is usually low, only ~50% (Yu and Hang, 1989 and Garg and Hang, 1995). Therefore, the fungal fermentation previously studied was done in submerged culture using glucose as a substrate. This is mainly because starch has relatively low solubility in water and is difficult to handle in the typical stirred tank fermentor. To minimize the problem found in the stirred tank fermentor, various methods, including cell immobilization, to control cell morphology and to achieve higher cell density, product yield, and production rate have been studied. Sun et al. developed a double-layer reaction-diffusion model for continuous L-lactic acid production with immobilized *R. oryzae* in an air-lift reactor (ALR) (Sun et al., 1999). In a pseudo-steady state, the productivity of lactic acid increased with the increasing dilution rate or feeding glucose concentration. Tay and Yang developed a cotton cloth carrier for *R. oryzae* in a fibrous bed, and lactic acid yield reached 90% with glucose and 100% with starch as substrates under repeated batch and fed-batch conditions (Tay and Yang, 2002)]. Efremenco et al. immobilized *R. oryzae* in a poly(vinyl alcohol) cryogel (PVA-cryogel), a macroporous carrier. Under the batch operation conditions, the lactic acid yield reached 94% with a productivity of 5 g/L·h in the shake flasks (Efremenco et al., 2006). In addition, by improvement of production media and cultivation condition, higher product yield and productivity

can be achieved. Wang et al. found that an increase in phosphate concentration from 0.1 to 0.6 g/L KH_2PO_4 reduced the maximum concentration of L-lactic acid from 85 to 71 g/L and fumaric acid from 1.36 to 0.18 g/L (Wang et al., 2005), whereas Zhou et al. indicated that an increase in the initial phosphate concentration from 0.2 to 1.6 g/L KH2PO4 had little effect on either biomass formation or lactic acid accumulation (Zhou et al., 1999).

However, enhancement of lactic acid production from the aforementioned methods still cannot provide up to a 100% yield due to the formation of by-products, including ethanol and fumaric acid. In the metabolic pathway of *R. oryzae*, pyruvate produced at the end of the glycolysis is channeled to lactate, ethanol, acetyl-CoA, and fumaric acid, depending on the fermentation condition (Longacre et al., 1997 and Wright et al., 1996). Several key enzymes, including pyruvate decarboxylase (PDC), pyruvate carboxylase (PC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and fumarase, are required for production of these metabolites. These enzymes play key roles in driving biochemical reactions, consequently determining the yield of the target product and proportion of final products (Zhang et al., 2007). When the key enzymes are regulated, lactic acid production can be enhanced and the by-products formation is limited. It was found that ethanol was produced as a major by-product in immobilized cell fermentation by *R. oryzae* although cell immobilization helped improve morphological control, mixing, and oxygen transfer (Thongchul, 2005).

Considering the metabolic pathway of *R. oryzae*, it was found that two enzymes involve in ethanol fermentative pathway. Those are PDC which converts pyruvate to acetaldehyde and ADH which couples with NADH to convert acetaldehyde to the end product ethanol. Therefore, in this study, the ethanol fermentative pathway of *R. oryzae* NRRL395 was manipulated by regulating these two enzymes. In vivo regulation of PDC and ADH by using the potential regulators obtained from the literature review was carried out. To obtain the targeted regulators, the pre-selected regulators were tested for their concentration effect on the activities of LDH, ADH, and PDC and fermentation kinetics in a shake flask culture. The results from the shake flask culture were applied in the fermentation of immobilized *R. oryzae* in a static bed bioreactor to study the coherent effect of dissolved oxygen and the presence of the enzyme regulators. It is believed that the results in this study will be helpful for developing the industrial lactic acid fermentation by *R. oryzae*.

CHAPTER II

THEORETICAL AND LITERATURE REVIEWS

2.1 Lactic acid

It is well known that lactic acid ($C_3H_6O_3$) is an organic acid which is widely applied in food and non-food industries. It was first found in sour milk by a Swedish chemist in 1780. Later, in 1881, it was first produced in a commercial scale in Massachusetts, USA. In recent years, the demand for lactic acid was evaluated to grow yearly at 5-8%. The worldwide production of lactic acid has been found to reach approximately 2590,000 metric tons in the year of 2012; moreover, it has been forecasted to be a trend in which production will continue to grow in the future (Abdel-Rahman et al., 2013).

2.2 Lactic acid properties

Lactic acid is 2-hydroxycarboxylic acid which consists of a hydroxyl and carboxyl group adjacent to the central carbon atom. It is a weak acid which exhibits a low volatility. Additionally, it partially dissociates in water, resulting in ion lactate and H^+. In its natural state, lactic acid exists in two optical isomers, including L(+)-lactic acid and D(-)-lactic acid (fig. 2.1).

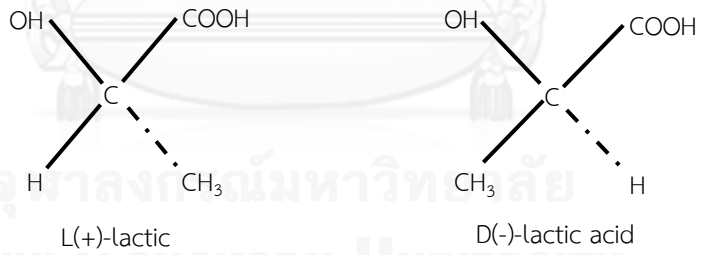


Figure 2.1 Structure of L(+) and D(-)-lactic acid

There is a difference betwen the boiling and melting point. Thereby, L(+)-lactic acid exhibits the boiling and melting point lower than D(-)-lactic acid (Budavari et al., 1989). D(-)-lactic acid is harmful to human metabolism; therefore, L(+)-lactic acid is a preferred isomer form in food and pharmaceutical industries. The other lactic acid properties are summarized in table 2.1.

Table 2.1 Chemical and physical properties of lactic acid

CAS registry number	79-33-4 (L-lactic acid) and 10326-41-7 (D-lactic acid)
Chemical formula	$C_3H_6O_3$
Chemical name	2-hydroxypropionic acid
Molecular weight	90.08 g/mol
pK_a at 25 °C	3.86
pH (0.1% solution, 25 °C)	2.90
pH (0.1N solution, 25 °C)	2.40
Physical appearance	Aqueous solution, colorless to slightly yellow
Melting point	L:53 °C; D:53 °C; D/L: 16.8 °C
Boiling point	122 °C at 12 mm Hg
Solubility in water (g/100 g H_2O)	Miscible

Source: Thongchul, 2013.

2.3 Application of lactic acid

Lactic acid is generally recognized as safe, or GRAS, and was approved by the US Food and Drug Administration (FDA) and European regulatory authorities. Of the demand for lactic acid, approximately 85% is utilized in food and food-related industries because it is a component in dairy products, while the non-food uses make up approximately 15% of the demand (John et al., 2007 and Martinez et al., 2013). Nowadays, it can be a monomer for producing biodegradable and biocompatible polylactic acid (PLA) which is becoming a sustainable plastic; therefore, the current market for producing lactic acid is expanding.

Food industry Lactic acid is used as acidulant and flavoring in a variety of food and beverage products. Due to its low pH, it can be used as a food preservative for preventing bacterial spoilage. Additionally, lactic acid is applied in bakery products by using the esters of calcium and sodium salts of lactate as dough conditioners and emulsifiers.

Cosmetic industry Lactic acid has long been used as an active compound in skincare products. It can be used as humectants and moisturizer agents which improve the skin texture and hydration level. Moreover, lactic acid has the ability to mildly exfoliate and cleanse the skin which is helpful to reducing skin tones and

smoothing the skin. It also functions as a pH adjuster by adding it to the product for making a suitable pH in cosmetic formulation. In addition, its derivative ethyl lactate is used as an ingredient for anti-acne products.

Pharmaceutical and medical industry Lactic acid has been mainly applied in pharmaceutical formulas; its derivatives include sodium and calcium salts. The sodium salt of lactic acid is used in parenteral and dialysis application. Lactic acid in the form of calcium salt can be used as a substance for calcium deficiency therapy and as an effective anti-carrying agent. Due to its ability to be a polymer, lactic acid can be a monomer for the production of polylactic acid (PLA) which has become an important sustainable plastic with the advantages of being a biodegradable and biocompatible material. From its polymer property, in medical applications, it is used as in surgical sutures, prostheses, and controlled drug delivery systems.

Chemical industry Lactic acid and its ester can be a feedstock monomer for the chemical conversion, including acetic acid, acrylic acid, propionic acid, etc. Additionally, lactate ester is an environmental friendly green solvent which can be applied in electronics and precision cleaning, including pesticides and bioactive compounds. Additionally, lactic acid can be an intermediate for the production of oxygenated chemicals.

Polymer industry In the recent years, the lactic acid polymer technology is widely commercially produced. Lactic acid is used as a precursor for the synthesis of polylactic acid (PLA). PLA is a biodegradable and biocompatible polymer which provides an environmentally friendly alternative to plastics derived from petrochemicals. Because of the high strength and thermo plasticity properties of PLA, it can be applied in product packaging, textiles, and medicals products. Furthermore, the wide range of PLA applications results in a great potential for expanding the market of lactic acid production.

2.4 The production of lactic acid

Lactic acid can be produced by either chemical synthesis or microbial fermentation. Both have been produced commercially by various companies.

2.4.1 Chemical synthesis

The chemical reaction for synthesizing lactic acid is based on petrochemical resources with the hydrolysis of lactonitrile by strong acids. The

reaction is started by mixing hydrogen cyanide and acetaldehyde under high atmospheric pressure to produce lactonitrile (eq.1). Subsequently, recovery and purification of the crude lactonitrile is done by using a distillation technique. The purified lactronitrile is hydrolyzed to lactic acid with strong acids, i.e. sulfuric acid or hydrochloric acid (eq. 2). Then methanol is used for esterification of lactic acid to produce methyl lactate; later, the methyl lactate is purified by distillation and hydrolyzed with water, occurring in lactic acid and methanol (eq.3 and 4). In addition, the chemical synthesis results in a racemic mixture of D(-) and L(+)-lactic acid which exhibits the limitation of lactic acid application in the industries; polymer application, an optical purity of L(+)-lactic acid is an especialy preferred isomer form due to its properties (Narayanan et al., 2004; Datta and Henry, 2006, and Wee et al., 2006).

(1) Addition of HCN

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Hydrogen cyanide}}{HCN} \xrightarrow{\text{catalyst}} \underset{\text{Lactonitrile}}{CH_3CHOHCN}$$

(2) Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH_3CHOHCN} + H_2O + \tfrac{1}{2}\underset{\text{Sulfuric acid}}{H_2SO_4} \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \tfrac{1}{2}\underset{\text{Ammonium salt}}{(NH_4)_2SO_4}$$

(3) Esterification

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH} \longrightarrow \underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O$$

(4) Hydrolysis by H_2O

$$\underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH}$$

2.4.2 Microbial fermentation

The chemical synthesis has the limitation to produce lactic acid by requiring the petrochemical resources such as crude oil or natural gas; furthermore, the chemical reaction exhibits an undesired stereoisomer of lactic acid under the drastic conditions, contributing to uneconomical and environmentally harmful effects (Thongchul, 2013). Therefore, the microbial fermentation is an alternative method for

commercially producing lactic acid. The lactic acid producer strain can be divided in two main groups which are lactic acid bacteria (LAB) and the filamentous fungi *Rhizopus*. Either an optically pure isomer or the mixture of D(-) and L(+)-lactic acid is obtained from the fermentation, depending on strain of microorganisms, substrates, and the cultivation conditions (Zhang et al., 2007).

2.4.2.1 Lactic acid bacteria

Most of the commercial lactic acid production and lactic acid bacteria (LAB) have been widely used because of their high product yield and growth rate. LAB are classified in gram-positive microorganism which can be divided in homofermentatives and heterofermentatives based on the fermentation end metabolites (fig. 2.2). The homofermentative bacteria are the favored group for the commercial lactic acid production because of its single fermentation end product, i.e. lactic acid, which converts 1 mol of glucose to 2 mol of lactic acid (1 g lactic acid/g glucose). The heterofermentative bacteria exhibits the byproducts with the lower lactic acid yield when compared to the homolactic acid bacteria (0.5 g lactic acid/g glucose). In addition, some of the heterofermentative LAB can be converted to pentose sugar which is obtained from the hydrolysis of hemicelluloses components of agricultural products to produce lactic acid (Wee et al., 2006; John et al., 2007; Litchfield, 2009; and Abdel-Rahman et al., 2013).

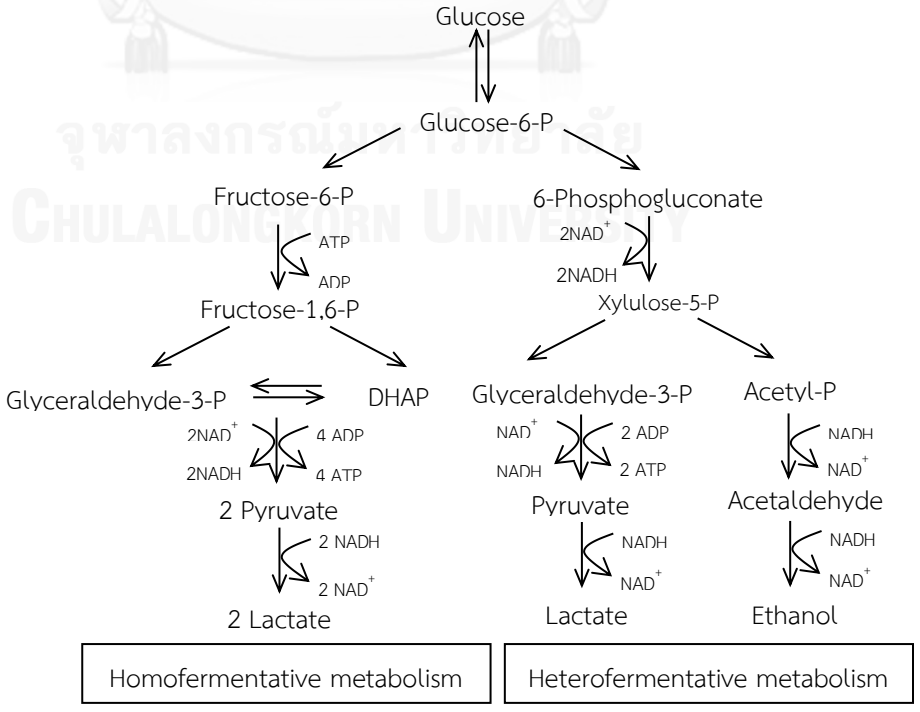


Figure 2.2 Metabolic pathways of lactic acid bacteria (Thongchul, 2013).

Many strains of lactic acid bacteria have been used as a lactic acid producer; particularly, *Lactobacilli* strains are important LAB species that have the ability to tolerate a high acid environment and they can be engineered for achieving the desired lactic acid isomer (Abdel-Rahman et al., 2013). Various studies have worked on the production of lactic acid by using LAB strains. Yun et al. (2003) have used *Enterococcus faecalis* RKY1 as a lactic acid producer. They compared the cultivation conditions by varying the types of carbon sources for cultivating bacteria, including glucose, fructose, maltose, galactose, starch, xylose, whey, and glycerol. They found that this LAB strain poorly utilized starch, xylose, whey, and glycerol, whereas using galactose as a carbon source, formic acid and acetic acid were found as the major products. Contrary to glucose, fructose, and maltose as a carbon source, *E. faecalis* RKY1 produced an optically pure L(+)-lactic acid through the homofermentative pathway with the high lactic acid yield of 0.96 g/g carbon source and average productivity in the range of 5.2-6.0 g/L·h (Yun et al., 2003). In addition, *Lactobacillus casei* was studied in fed-batch fermentation in glucose medium for producing L(+)-lactic acid. The maximum of L(+)-lactic acid concentration of 180 g/L was obtained from the exponential feeding of glucose and yeast extract with a yield of 90.3 % and productivity of 2.14 g/L·h which was higher than the batch fermentation. This indicated that controlling nutrient concentration is possible to improve product formation by increasing logarithmic phase of cell growth (Ding and Tan, 2006). A new sub-species of *Lactobacillus paracasei* was found by Moon et al. (2012). *Lactobacillus paracasei* subsp. *paracasei* CHB2121was isolated from soil nearby the ethanol production factory. This new strain efficiency produced 192 g/L lactic acid with 0.96 g/g glucose and 3.99 g/L·h. Besides, the new identified strain produced L(+)-lactic acid in the optical purity of 96.6% (Moon et al., 2012).

Although lactic acid bacteria efficiently produces lactic acid, it suffers from many limitations. Because LAB strains have both D(-) and L(+) lactate dehydrogenase activity which responds to produce D- and L(+)-lactic acid which overcomes metabolic engineering problem. Furthermore, the formation of byproducts leads to low lactic acid yield; hence, LAB is needed to manipulate their metabolic pathways. In addition, due to their biosynthetic inability, the complex nutrient is required for cell growth and lactic acid production, i.e. vitamins, amino acids, nucleotides, and peptides, which impact product recovery and purification and also increases production costs (Litchfield, 2009; and Abdel-Rahman et al., 2013).

2.4.2.2 Filamentous fungi

Nowadays, the filamentous fungi *Rhizopus* species have been extensively studied as a lactic acid producing strain because of its advantageous ability. The genus *Rhizopus* can be a candidate for producing lactic acid compared to LAB, particularly the optically pure L(+)-lactic acid production. Among the different *Rhizopus* strains, *Rhizopus oryzae* is the most favored strain that provides great lactic acid production (Soccol et al., 1994; Yin et al., 1997; Oda et al., 2003; and Thongchul, 2013). *Rhizopus oryzae* is recognized as a suitable lactic acid producer because it can grow well and produce L(+)-lactic acid in a simple medium, leading to a simple and cheap downstream process. Furthermore, it has the ability to utilize agricultural residues such as sugarcane, cassava pulp, corn corps, and paper pulp sulfite liquor as carbon sources. (Zhang et al., 2007; Litchfield, 2009; and Abdel-Rahman et al., 2013). In addition, many studies discovered that *R. oryzae* can be protease, urease, ribonuclease, pectate lyase, and polygalacturonase producers (Ghosh and Ray, 2011).

2.5 *Rhizopus oryzae*

Filamentous fungi *Rhizopus oryzae* is classified in the kingdom of fungi, order of mucorales, and mucoraceae family. It has many synonyms such as *R. arrhizus, R. javanicus, R. japonicas, R. thermosus, Rhizopus schizans*, etc. *R. oryzae* has been isolated from soil and plant materials. Its colonies' color is grayish to brownish which can be found as bread mold (fig 2.3). Its hyphae are tube-like, without septation, and the cell wall contains chitosan and chitin instead of glucans, mannans, and chitin which are different from ascomycetes and basidiomycetes. This fungus belongs to the zygomyces group, which uses zygospore and sporangiospore as sexual and asexual reproduction. In sexual reproduction, sporangiospores (non-motile spores) are produced and released from sporangia when they are maturative. With sexual reproduction, the fungus has a bipolar mating system which consists of positive and negative mating types and subsequent production of zygospores (Gryganskyi et al., 2010). In addition, *R. oryzae* has the potential to produce a wide range of metabolites, including enzymes, ester, organic acids, volatile materials, polymers, and bioalcohols. This fungus strain is particularly recognized as the commercially perspective producer of L(+)-lactic acid (Ghosh and Ray, 2011).

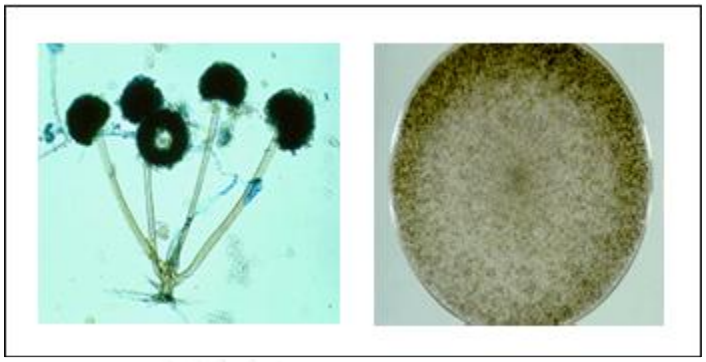


Figure 2.3 Characteristic of *R. oryzae* (Ellis, 1997).

2.6 *Rhizopus* metabolism

In glucose metabolism of *Rhizopus oryzae*, it is found that at the pyruvate branch point, pyruvate can be channeled to several metabolic routes, depending on the cultivation condition (Fig. 2.3) (Zhang et al., 2007). The pools of pyruvate in *R. oryzae* separate into 2 metabolic pools, including cytosol and mitochondria (Wright et al., 1996). The mitochondrial pools is responsible for fungal growth and energy production. Pyruvate is oxidized to acetyl-CoA, NADH and CO_2 by the reaction of pyruvate dehydrogenase (PDH) complex PDH is a complex enzyme which consists of 3 main enzymes, including pyruvate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2), dihydrolipoyl dehydrogenase (E_3). These complex enzymes require TPP, lipoate coenzyme A, and FAD as their cofactors, respectively. Sequentially, acetyl-CoA is further entered into tricarboxylic acid (TCA) cycle for generating ATP and NADH.

The cytosolic pools exhibit various metabolites, i.e. lactic acid, ethanol, and fumaric acid. Under sufficient mass transfer in *R. oryzae*, 1 mol of glucose can be converted to 1.5 mol of lactic acid by the reaction of lactacte dehydrogenase (LDH). LDH (EC 1.1.1.27) is an enzyme which requires NADH as a cofactor for converting pyruvate to L(+)-lactic acid. This enzyme has two NAD^+-dependent LDH isozymes which encode 2 genes, *Ldh*A and *Ldh*B. At the initial cell growth and synthesis of lactic acid, NAD^+-dependent LDH encoding *Ldh*A responds to convert pyruvate to L(+)-lactic acid until the glucose level is depleted which is without the activity of NAD^+-independent LDH encoding *Ldh*B. NAD^+-independent LDH encoding *Ldh*B involves in the reverse reaction by converting L(+)-lactic acid to pyruvate, which can activate in the culture containing non-fermentable substrates such as glycerol and ethanol. In addition, when glucose is exhausted, it results in a decrease of lactic acid

levels; later, lactic acid levels are increased. This is perhaps because *Ldh*B could activate for this reaction (Pritchard, 1971; and Skory, 2000).

Another metabolic route is ethanol, where two enzymes are involved in ethanol production, including pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). In an O_2 limiting environment, pyruvate is converted to acetaldehyde by the reaction of PDC. PDC (EC 4.1.1.1) is an isoenzyme which requires thiamine pyrophosphate (TPP) as co-factors, and it has two genes which are *pdc*A and *pdc*B. This gene can be induced by the addition of glucose and an anoxic condition. Moreover, PDC is a key enzyme in the regulation of ethanol production and the support of respiration by supplying acetyl CoA to the TCA cycle (Açar et al., 2007). Consequently, acetaldehyde is converted to ethanol by alcohol dehydrogenase (ADHs). *Rhizopus* ADHs (EC 1.1.1.1) are oxidoreductase enzymes which consist of two ADH isoenzymes, including ADH I and ADH II. ADH I is a fermentative enzyme which is related to the ethanol formation. ADH II is less expressed in the oxidative metabolism of ethanol (Yoneya and Sato, 1979; and Skory, 2003). The active site of ADH enzyme contains one reactive sulphydryl group (SH-group), one of zinc atom, and requires NADH/NAD^+ as co-factors for catalytic function (Smidt et al., 2007). In addition, *Rhizopus* ADH was found to have the similarity of conformation to yeast ADH (Yoneya and Sato, 1979).

Regarding fumaric acid production in *R. oryzae*, it is produced in the cytosol by combining activities of pyruvate carboxylase, malate dehydrogenase, and fumarase (Skory and Ibrahim, 2007). Interestingly, there was a report of the two-group classification of *R.oryzae* which are Type I strains that primarily produce lactic acid and type II strains that produce a fumaric acid in the major product. The difference between type I and II is the Ldh coding gene which means that *Rhizopus* type I contains both *Ldh*A and *Ldh*B genes, while type II only has the *Ldh*B gene (Saito et al., 2004). Furthermore, the predominance of fumaric acid in type II strains has been suggested as lacking the capability for reductive of pyruvate to lactic acid, contributing to excess pyruvate would be available for producing fumaric acid or ethanol, thus serving as an alternative generating NAD^+ route (Skory and Ibrahim, 2007).

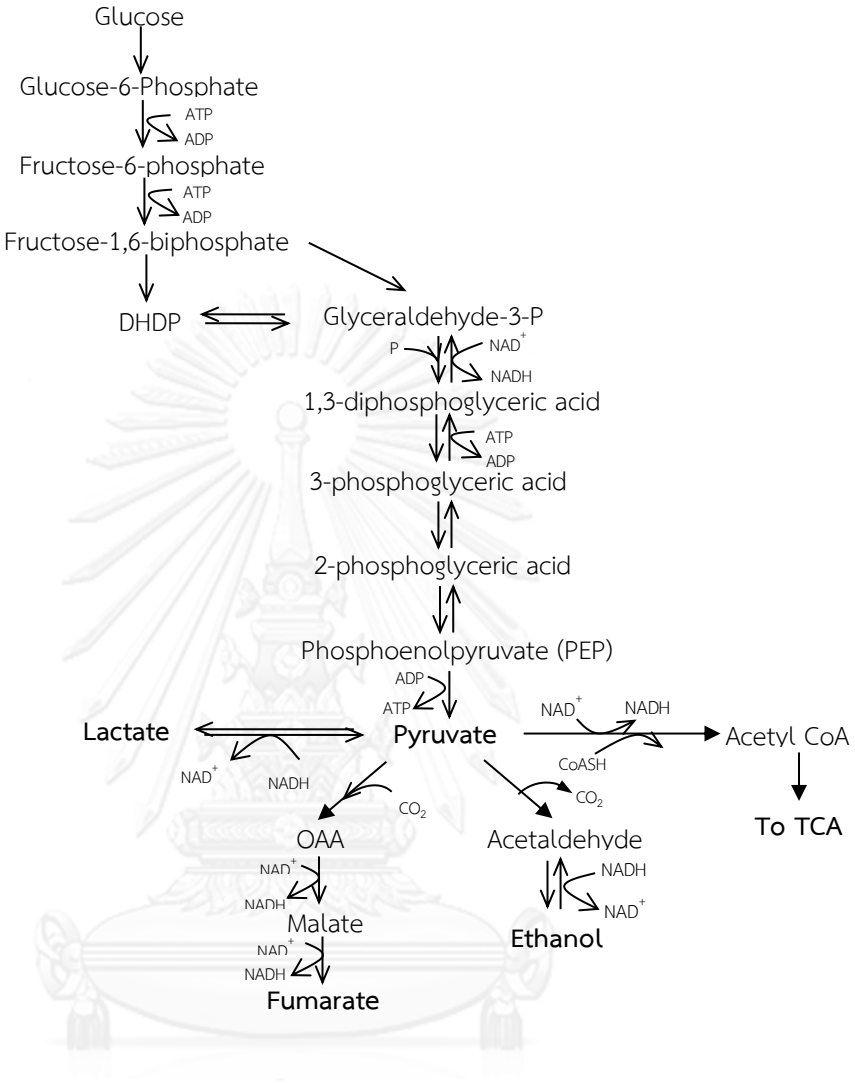


Figure 2.4 Glucose metabolism of *Rhizopus* (Thongchul, 2013).

2.7 Limitation of using *R. oryzae*

Many previous research studies have found the main problems of L(+)-lactic acid by using *Rhizopus* species is less lactate yield and productivity, compared to LAB. Instead of lactic acid production, some byproducts can be found in *Rhizopus* metabolism, including ethanol and fumaric acid. For lactic acid producers, *R. oryzae* is usually found undesirabe because of byproduct formation, i.e. ethanol. The formation of product can be influenced by several fermentation parameters, including pH, medium composition, neutralizing agent, fungal morphology, and oxygen transfer (Zhang et al., 2007). All of these parameters are related, thus to manage one or more such parameter, resulting in the formation of a desired product. In addition, all metabolites are formed by their enzymes activity. This indicates that all of these parameters are able to affect enzyme activity.

2.7.1 Fungal morphology and cell immobilization

One factor which plays an important role and impact on lactic acid production is fungal morphology because of its morphological changes, contributing to a decrease in fermentation broth rheology; hence, limitation of mass transfer will occur. The change of fungal morphology depends on inoculum sizes, medium composition, pH controller, agitation speed, and aeration rate. A variety of fungal morphology has been found, including freely dispersed mycelia, fluffy pellets, compact pellets, and dense pellet (clumps). In free cell fermentation, loosely dense pellets are a preferred form because oxygen and nutrients can diffuse into the center of the compact cell. However, to form the loosely dense pellets in the bioreactor, the cultivation condition is necessary to control (Papagianni, 2004; Zhang et al., 2007; and Krull et al., 2013). Alternatively, cell immobilization technique has been used for improving oxygen transfer in fungal fermentation. This technique has been widely used for controlling fungal morphology on cell supporters. Various polymeric carriers have been used as cell supporters, including calcium alginate, loofa sponge, cotton cloth, polyurethane foam, mineral, and polymer support. Fungal morphology is immobilized on the carrier by cell entrapment, cell attachment, and also itself-immobilization on the carrier surface. The advantage of this technique is it is able to improve mass transfer inside the starvation zone of fungal mycelia and fermentation broth, leading to achieve high cell density and increase cell productivity; thus, enhancement of lactic acid production is obtained. In addition, this technique is easier to operate and control cell morphology in bioreactor, providing fermentation broth is easy to separate from fungal cell. Additonally, immobilized cells can be

used in long term fermentation by cell recycling (Tay and Yang, 2002, Wang et al., 2010, Chotisubha-anandha et al.,2011).

Prichard (1971) reported *R. oryzae* mycelium contains two lactate dehydrogenases depending on the state of cell growth and glucose concentration. An NAD^+-dependent lactate dehydrogenase which catalyses the reduction of pyruvate to lactate is produced during initial cell growth and glucose utilization. After glucose is exhausted, the first LDH is replaced by a second NAD^+-independent lactate dehydrogenase catalyzing the oxidation of L-lactic acid to pyruvic acid (Prichard, 1971).

Büyükkileci et al. (2006) investigated the activities of related pyruvate branch point enzymes in *Rhizopus oryzae* ATCC 9363. They found that the inoculum size influenced the morphology and enzyme activities in *R. oryzae* in the shake flask cultures. Inoculum concentrations of 1×10^3 spore/mL induced one large tightly packed clump and gave the highest lactate, lowest ethanol, and lowest biomass concentration. That is in contrast with an inoculum size of 1×10^6 spores/mL, it gave the lowest lactate, highest biomass, and highest ethanol productions. Enzyme activities were determined in 100 mL cultures inoculated with either 1×10^3 or 1×10^6 spores/mL which maximized lactate or ethanol production, respectively (Büyükkileci et al., 2006).

Wu et al. (2011) studied the L(+)-lactic acid production in semicontinuous fermentation by varying the seed culture and sporing suspension size of *R. oryzae* from 2-30 % and 2-12%, respectivly. Variation of *R. oryzae* morphology was formed depending on the inoculums' size. The pellets form of *R. oryzae* gave the highest lactic acid production. A large inoculums size resulted in clump floc and clump form, and decreased the L(+)-lactic acid production in *R. oryzae.* Moreover, the semicontinuous fermentation for 25 cycles using pellets of *Rhizopus oryzae* was carried out in flask culture. In addition, no change was observed in the lactate dehydrogeanse (LDH) activity in 19 repeated fermentation cycles. In the last five cycles (cycles 21–25), the pellet was ruptured, and fungi autolysis led to the metabolic capability of *R. oryzae* decrease, resulting in the decrease of L(+)-lactic acid production in these fungus strains (Wu et al., 2011).

Chotisubha-anandha et al. (2011) determined the relationship between the shear effects (agitation and aeration) and *R. oryzae* morphology and metabolism, lactate dehydrogenase activity in L(+)-lactic acid fermentation by using free cell and

immobilized *Rhizopus oryzae*. They found that the maximum lactic acid production and a lower enzyme activity were observed in the immobilized *R. oryzae* culture compared with free cells operating in the same conditions. From this phenomenon, due to the mass transport in immobilized cells higher than the reaction rate in free cell cultures, it could be concluded that mass transport dominated *R. oryzae* metabolism; thus, it controlled the apparent production rate (Chotisubha-anandha et al., 2011).

2.7.2 Oxygen supply

The submerged L(+)-lactic acid fermentation by using *Rhizopus oryzae* requires aerobic fermentation. Oxygen supply is a chief requirement for cell growth and L(+)-lactic acid production. Aeration and agitation are the oxygen ventilation factors in a fermentor which directly affects dissolving oxygen and the shearing rate in the fermentation process (Thongchul, 2005). From the above mentioned, L(+)-lactic acid fermentation by using *R. oryzae* requires oxygen transfer. By means of adequate oxygen, the transferring rate leads to enhancement of L(+)-lactic acid production in this fungus strain. However, *Rhizopus oryzae* has a complex and various morphology which is difficult to control in bioreactor cultivation. A variation of fungi morphology leads to an increase in broth viscosity and reduces oxygen transferring inside the mycelia clump or pellets. Furthermore, under insufficient oxygen transferring in fungal fermentation, by-product especially ethanol is formed, result in a low yield, and productivity of L(+)-lactic acid is produced in this fungus strain (Tay and Yang, 2002). In recent years, several works of literature have been studied on the development of L(+)-lactic acid production in *R. oryzae* by controlling their morphology including pellet form. Additonally, cell immobilization is the method that is used for improving L(+)-lactic acid production in this fungus strain. However, the controlling *R. oryzae* morphology is also influenced from aeration and agitation. It is well-known that NAD^+-dependent lactate dehydrogenase (LDH) is a lactic acid producing enzyme in *R. oryzae* that requires NAD+ and NADH as a co-factor and which converts pyruvate to L(+)-lactic acid (Pritchard, 1971 and 1973). The aforementioned ethanol is produced under oxygen limiting conditions. Pyruvate is converted to acetaldehyde by pyruvate decarboxylase (PDH) and the conversion of acetaldehyde to ethanol by alcohol dehydrogenase (ADH) (Açar et al., 2007), thus it could be implied that enzyme activities can be affected by shear effect. However, little research states any relationship between shear effect and enzyme activities.

Skory et al. described L(+)-lactic acid production in *Rhizopus oryzae* being produced during aerobic condition, thus pyruvate is converted to L(+)-lactic acid by lactate dehydrogenase (LDH). While limiting condition oxygen, ethanol is a primary product. In addition, a higher alcohol dehydrogenase than lactate dehydrogenase activity was observed from oxygen limiting condition (Skory, 1998).

Bai et al. observed that the increase of agitation speed and aeration rate led to an increased biomass concentration and lactic acid concentration. The increase agitation speed resulted in small pellet growth of *R. oryzae* and enhanced shear stress in this fungus strain. Besides, the aeration rate led to enhancement of dissolving oxygen and increased in size of fungal pellets but to decrease in number per unit volume (Bai et al., 2003).

Zhang et al. concluded that dissolving oxygen (DO) plays an important role on cell growth and L(+)-lactic acid production in *Rhizopus* species. A high productivity and yield of lactic acid were achieved from a high DO level. In addition, an over low and high shear stress may inhibit mycelial growth at an early cell growth phase and prolong the lactic acid production period and result in low productivity (Zhang et al., 2007).

Bulut et al. found that maximum lactic acid concentration was obtained at an agitation rate of 150 rpm, whereas at high agitation speed of 250 rpm showed the negative effect on cell growth of *Rhizopus oryzae*. On the other hand, insufficient oxygen supplies for cell growth, and product formation was observed in a low agitation rate of 75 rpm (Bulut et al., 2009).

Yadav et al. stated that the aeration and agitation should be combined for aerobic *Rhizopus species* fermentation in the bioreactor. Due to the limited oxygen, it can promote the ethanol production in *Rhizopus oryzae* metabolism (Yadav et al., 2011).

2.7.3 pH

pH is one important factor to cell growth and L(+)-lactic acid production in *R. oryzae*. It is recognized that a favorable pH range is 5.0-6.0 (Zhang et al., 2007). Miura et al. reported that pH 6.5 gave the highest L(+)-lactic acid production in 3 L airlift bioreactor (Miura et al., 2003). Like Tay and Yang, they found that a faster fermentation and more produced lactic acid at pH 6 than did the lower

pH value (Tay and Yang, 2002). Bulut et al. reported the highest lactic acid concentration was obtained from pH 6. Moreover, they found that the high pH level led to the culture environment be in the stressful condition, which is too acidic and the alkaline resulting in reducing lactic acid production (Bulut et al., 2009). Furthermore, Yu et al. reported the improvement of *R. oryzae* mycelia bioactivity was obtained from pH 5.5. However, at pH more than 5.5 led to the converting of glucose into biomass and dissipated energy, in turn reducing conversion rate of lactic acid (Yu et al., 2007).

2.7.4 Medium composition

The advantage of L(+)-lactic acid fermentation by *R. oryzae* requires a small amount of inorganic salts. The main inorganic salts are used in lactic acid production media including KH_2PO_4, $MgSO_4$, and $ZnSO_4$. Normally, in a range of 0.15-0.60 g/L KH_2PO_4, 0.15-0.75 g/L $MgSO_4 \cdot 7H_2O$ and 0.04-0.09 g/L $ZnSO_4 \cdot 7H_2O$ is used (Zhang et al., 2007). Zhou et al. found the initial phosphate concentration in the form of KH_2PO_4 from 1.5–12 mM has no significant effect on either biomass or lactic-acid accumulation. Furthermore, when phosphates were neglected, *Rhizopus* spores failed to grow beyond the germinating stage (Zhou et al., 1999). In addition, Zhou et al. investigated the effect of magnesium, zinc, iron, copper, and manganese which were contained in the medium on pellet formation in *R. oryzae* ATCC 20344. They found that spores of *R. oryzae* could not germinate when magnesium was not present in the cultivation media. Similar to the absence of zinc, *R. oryzae* that could not form in pellet morphology, they concluded that magnesium and zinc were essential for cell growth and pellet formation in this fungus strain. Also, iron and manganese resulted in affecting pellet formation and spore germination respectively. They observed that iron did not make a great contribution to the formation of small pellets. Moreover, no spore germination in the presence of manganese concentration was more than 100 ppm in the cultivation medium (Zhou et al., 2000) The higher yields of lactic acid were obtained from the poor medium compositions and cultivation conditions which was studied by Taherzadeh et al., whereas the ethanol and biomass yields were higher in rich media. Furthermore, they found that the addition of a large amount of zinc sulphate did not stimulate the growth of *R. oryzae*; therefore, zinc was not essential for cell growth (Taherzadeh et al., 2003). Wang et al. concluded that a high level of KH_2PO_4 in the medium could promote both mycelial growth and the consumption of substrate (glucose). Consequently, it reduced the amount of by-product, especially fumaric acid, which benefits the

enhancement of L(+)-lactic acid production. Despite the slight decrease, L(+)-lactic acid yield was obtained from increasing phosphate concentration, there are economic methods for the production of L(+)-lactic acid; for example, the higher volumetric productivity, the shorter time of fermentation and lower quantity of byproduct (Wang et al., 2005).

2.8 Improvement of lactic acid production

Lactic acid is an important organic acid which is extensively used in several industries. It can be produced by either chemical synthesis or microbial fermentation. In recent years, filamentous fungi *R. oryzae* has become an alternative lactic acid producing strain due to its many abilities, including solely L(+)-lactic acid production, amylolytic enzyme activity, requiring the simple media for growth and product formation, and it is acid tolerant. However, it is a filamentous fungus which has a complex morphology and difficult to control in the fermentor leading to low lactic acid yield and productivity. Therefore, using *R. oryzae* as lactic acid producer in submerge fermentation, *R. oryzae* should improve its limitations. Several methods have been used for improving lactic acid production, including bioprocess parameters optimization and metabolic engineering.

2.8.1 Bioprocess parameters

As mentioned about the parameters effect on lactic acid production, there are many parameters impact on cell growth and product formation, including pH, medium composition, cell immobilization, oxygen transfer, and fermentation system. Most research has found that the improvement of lactic acid production by optimizing such parameters could enhance *R. oryzae* performance to increase L(+)-lactic acid formation. However, none of this research could complete limitations of ethanol formation in this fungus strain.

2.8.2 Metabolic engineering

In *Rhizopus* metabolism, several metabolites can be exported by the response of several key enzymes. Additionally, *R. oryzae* strains have been divided into 2 groups depending on the main product formation during growth in a glucose medium, i.e. lactic acid and fumaric acid producing strains. These 2 groups are different in the rDNA sequences. The used of lactic acid producing strains, unless it produces primarily lactic acid, ethanol can be also formed. Ethanol is the main byproduct which reduces lactic acid yield and productivity. Therefore, the genetic

engineering technologies become an alternative technique for minimizing the byproduct formation. The successful use of gene heterologous gene expression in *R.oryzae* was found in the year 2002 by Skory. Spores were transformed into the uracil auxotrophic strain derived from *R. oryzae* NRRL 395 by introducing the DNA with vector DNA which was coated with tungsten particles (Skory, 2002). Later, this system has been applied in improving the expression of holomologous genes such as *ldh*A (Skory, 2004). Moreover, RNA interference (RNAi) is widely interesting for genes expression. This technique affects the diminishing of protein translation. Gheinani et al. (2011) have used siRNA to reduce L(+)-lactic acid production in *R. oryzae* by silencing the *ldh*A and *ldh*B genes. They found the successful silencing of these genes which reduced the pyruvate flux towards L(+)-lactic acid and thereby increase the ethanol formation. However, the effect of gene silencing with siRNA is unstable; thus, it was not suitable for an industrial process (Gheinani et al., 2011). Furthermore, the genetic engineering technology is still not acceptable in many developed countries due to unclear of its further effect (Yadav et al., 2011).

2.8.3 The inhibition of enzyme activity in ethanol fermentative pathway

As a result, this study attempted to improve L(+)-lactic acid production in *R. oryzae* by using the potential compounds to regulate the ethanol fermentative enzymes, i.e. PDC and ADH. Many compounds were selected from the literature reviews that have worked on the in vitro inhibition of yeast ADH. Yoneya and Sato purified and characterized ADH produced by *R. javanicus.* They found that ADH in *R. javanicus* was similar to yeast ADH (YADH). Both ADH in *R. javanicus* and YADH were inhibited by sulfhydryl reagents including iodoacetic acid, *N*-ethylmaleimide, and *p*-chloromercuribenzoate (Yoneya and Sato, 1979). In addition, it was found that pyrazole acted as an ADH inhibitor in yeast and the human liver. This inhibition was competitive in nature and could be overcome by increasing the amount of ethanol (Leskovac et al., 1997; Singlevich and Barboriak, 1970). Similarly, 2,2,2-trifluoroethanol acted as a competitive inhibitor of yeast ADH. 2,2,2-trifluoroethanol bound more tightly to YADH-NAD^+ complex than did ethanol, but no oxidation occurred (Leskovac et al., 1997; Taber 1998). Furthermore, YADH was inhibited by the presence of 4,4´-dithiodipyridine. The reaction was competitive and complex (Zheng et al., 1997).

Somehow it is believed that inhibition of ADH might not be sufficient to prevent pyruvate flux to ethanol fermentative pathway since PDC is still available in an active form. Several studies on YPDC and bacterial PDC have been conducted. Thomas et al. (1990) examined the various concentration of 3-hydroxypyruvate on

PDC activity of *Zymomonas mobilis*. The inactivation reaction was performed by using purified PDC; subsequently, 3-hydroxypyruvate was tested for the PDC inhibition effect. This compound is substrate analog with pyruvate which is a central substrate in microorganism metabolism. Therefore, the PDC inhibition by 3-hydroxypyruvate, this reagent was competitive with pyruvate (Thomas, et al., 1990).In addition, Wang et al. (2004) studied the in vitro inhibition of PDC in *Torulopsis glabrata* IFO005 by using glyoxylate as inhibitor. They investigated the effect of different concentrations of this compound on enzyme activity. It was indicated that glyoxylate was found to be a strong PDC which formed coenzyme ThDP with a non-cleavable bond at the catalytic site of this enzyme (Wang, et al., 2004). This study attempted to regulate PDC in the living culture of *R. oryzae* by using potential PDC regulator, however,this study has used the ADH regulator which is 4-methylpyrazole or fomepizole as PDC regulator. ADH plays an important role in acetaldehyde metabolism. In addition, this regulator is a competitive inhibitor of ADH 2 by binding at the active site of this enzyme. Most of this work has used this inhibitor as a drug for treatment of alcoholic patients (Mcmartin et al., 2012; Wang et al., 2013; and Sande and Thompson, 2012).

Chapter III

EXPERIMENTAL

3.1 Materials

3.1.1 Equipments and instruments

Instrument	Model	Company and country
Autoclave	KT-30 SD	ALP Co., Ltd., Japan
Microscope	Alphaphot-2	Nikon Co., Ltd., Japan
Haemacytometer	Neubauer Bright line	Bocco Co., LTD., Germany
Electronic balance	New Classic ML	Mettler Toledo, Switzerland
pH meter	AB15	Fisher Scientific Pte Ltd., Singapore
Rotary incubator shaker	Infors HT Bottmingen	Infors HT, Switzerland
Incubator	-	Sanyo, Japan
Laminar flow hood	NK system clean bench	International Scientific supply, Thailand
Spectrophotometer	Synergy-HT multimode Microplate Reader	Biotek, USA
Fermentor 5 L	MD 300-5L	B.E. Marubishi, Japan
Bio process controller	MD 300-5L	B.E. Marubishi, Japan
Air compressor	-	Hitachi, Japan
Circulation type hardy cooler	TRL-108	Thomas Kagaka, Japan

pH combination electrode	405-DPAS-SC-K85/225	Mettler Toledo, Switzerland
Dissolved oxygen electrode	O_2 sensor InPro	Mettler toledo, Switzerland
Vortex mixer	Vortex-genie No. 2	Scientific industries Inc., USA
Oven	UL-80	Memmert Co., LTD., Germany
Centrifuge	Mikro 200R	Hettich, Germany
Ultrasonic sonicator	UD-201	Tomy, Japan
High Performance Liquid Chromatography (HPLC)	10A*vp*	Shimadzu, Japan

3.1.2 Chemicals

Chemical	**Grade**	**Company and country**
Glucose ($C_6H_{12}O_6$)	Commercial grade	Siamchai chemical, Thailand
Yeast extract	Commercial grade	Bio Springer, France
Potassium dihydrogen phosphate (KH_2PO_4)	AR grade	Carlo Erba, France
Magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$)	AR grade	Carlo Erba, France
Zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$)	AR grade	Carlo Erba, France
1,2-diazole	AR grade	Sigma Aldrich, Germany
2,2,2-trifluoroethanol	AR grade	Sigma Aldrich, Germany
4,4-dithiodipyridine	AR grade	Fluka, Switzerland
Iodoacetic acid	AR grade	Fluka, Switzerland

p-chloromecuric benzoate	AR grade	Fluka, Switzerland
N-ethylmaleimide	AR grade	Fluka, Switzerland
4-methylpyrazole	AR grade	Sigma Aldrich, Germany
Glyoxylic acid	AR grade	Sigma Aldrich, Germany
Omeplazole	AR grade	Sigma Aldrich, Germany
β-hydroxypyruvate	AR grade	Sigma Aldrich, Germany
Sodium hydroxide (NaOH)	AR grade	Merck, Germany
Hydrochloric acid (HCl)	AR grade	Merck, Germany
Sulfuric acid (H_2SO_4)	AR grade	Merck, Germany
Dibasic phosphate	AR grade	Carlo Erba, France
Potato dextrose agar (PDA)		Himdia
Trizma® base buffer substance	AR grade	Sigma Aldrich, Germany
β-Nicotinamide-adenine Dinucleotide, Reduced, 2Na (NADH)	AR grade	Merck, Germany
β-Nicotinamide-adenine Dinucleotide (NAD^+, free acid)	AR grade	Merck, Germany
Acetaldehyde	AR grade	Sigma Aldrich, Germany
Absolute ethanol (C_2H_5OH)	AR grade	Merck, Germany
ADH from baker yeast	AR grade	Sigma Aldrich, Germany
Magnesium chloride ($MgCl_2$)	AR grade	Sigma Aldrich, Germany
Thiamine diphosphate	AR grade	Sigma Aldrich, Germany
Sodium pyruvate	AR grade	Fluka, Switzerland

Calcium L-lactic acid	AR grade	Fluka, Switzerland
Fumaric acid	AR grade	Fluka, Switzerland
Antifoam Y-30 emulsoin	AR grade	Sigma Aldrich, Germany

3.2 Microorganism and inoculums preparation

Rhizopus oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Agricultural Research Service Culture Collection, US Department of Agriculture, Peoria, IL, USA, was used in this study. The sporangiospores were collected from the 7-day culture on potato dextrose agar plate by shaving the surface with a sterile loop and extracting the spores with sterile water. The spore concentration was determined by spore counting using a haemacytometer (Appendix A). The spore suspension was diluted to 10^6 spores/mL using sterile water.

3.3 Fermentation medium

L(+)-lactic acid fermentation by *R. oryzae* were divided into 2 phases, i.e. growth and production phase. Growth medium consisted of 50 g/L glucose and 5 g/L yeast extract. This phase promoted spore germination and initial cell growth. To enhance L(+)-lactic acid production, production medium was replaced. Production medium contained 70 g/L glucose, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.088 g/L $ZnSO_4 \cdot 7H_2O$, and 0.3 g/L urea (Thongchul, 2005).

3.4 Effect of different enzyme regulators on fermentation behavior in shake flask culture

3.4.1 Growth of immobilized *R. oryzae* in the fermentation medium containing different enzyme regulators

Immobilization of *R. oryzae*, a preweighed cotton cloth (5 × 5 cm) was used for controlling fungal morphology. Spores suspension of 1 mL was inoculated into 250 mL Erlenmeyer flask contained 100 mL growth medium with cell supporter. After 48 h in growth phase, growth medium was removed and 100 mL production medium was added instead. During production phase, 10 M NaOH was added for pH control. Sample was taken every 6 h for analyses of the metabolites as well as enzyme assays.

3.4.2 Selection of potential regulators

The in vivo regulation of ethanol fermentative pathway of *R. oryzae*, there were 2 enzymes which were regulated, including ADH and PDC. The enzyme regulators were selected from the literature reviews that worked on the in vitro ADH and PDC inhibition of yeast and bacteria.

3.4.2.1 ADH regulators included pyrazole (1,2-diazole), 2,2,2-trifluoroethanol, 4,4'-dithiodipyridine, and sulfhydryl reagents, i.e., iodoacetic acid, *N*-ethylmaleimide, and *p*-chloromercuriobenzoate.

3.4.2.2 PDC regulators included glyoxylic acid, beta-hydroxypyruvate, 4-methylpyrazole, and omeprazole.

Firstly, all of enzyme regulators were tested the effect of type on fungal growth with the initial concentration of 1 mM. Except for β-hydroxypyruvate, it was tested at 1 μM. After screening those regulators which did not harm fungal growth and metabolism in the in vivo test, the concentration effects of such inhibitors were further studied.

In addition, due to the formation of ethanol during growth phase; therefore, to add the enzyme regulator in different fermentation phase were investigated. It was meant that the selected regulators were determined the effect on the fermentation routes by adding the regulator since growth medium and to add during production medium, including the fermentation medium without regulator (control).

3.5 Regulatory effects of 1, 2 – diazole and 2,2,2 – trifluoroethanol on metabolism of *R. oryzae*

The 2 – phase fermentation by *R. oryzae* was carried out in a shaken flask culture. The fermentation was initiated by inoculating 1 mL *R. oryzae* spore suspension (10^6 spores/mL) into 100 mL sterile growth medium in the 250 mL Erlenmeyer flask. The growth phase took 48 h before the growth medium was discarded and replaced by 100 mL sterile production medium containing either 1, 2 – diazole or 2, 2, 2 – trifluoroethanol at the concentration of 1 mM. The control fermentation was also carried out in the glucose medium under the same cultivation conditions but without the inhibitors. During the production phase, the pH of the culture was maintained at 6.0 by periodically adding 10 M NaOH. The production

phase was carried out until glucose was depleted. Samples were collected at every 8 h for analyzing the remaining glucose, L – lactic acid, and ethanol.

3.6 Fermentation behavior in 5 L static bed bioreactor with the presence of the potential enzyme regulators

3.6.1 Bioreactor construction

A static bed bioreactor was modified from a 5-L stirred tank bioreactor by affixing a fibrous matrix to the top plate of the bioreactor. The fibrous matrix was made of one sheet of cotton towel attached to the surface of a perforated stainless steel cylinder mounted on the top plate (Fig. 3.1) (Tay and Yang, 2002). The bioreactor was equipped with the automatic temperature, pH, agitation, and aeration control. The air fed into the bioreactor was sterilized by passing through a membrane filter (0.2 μm).

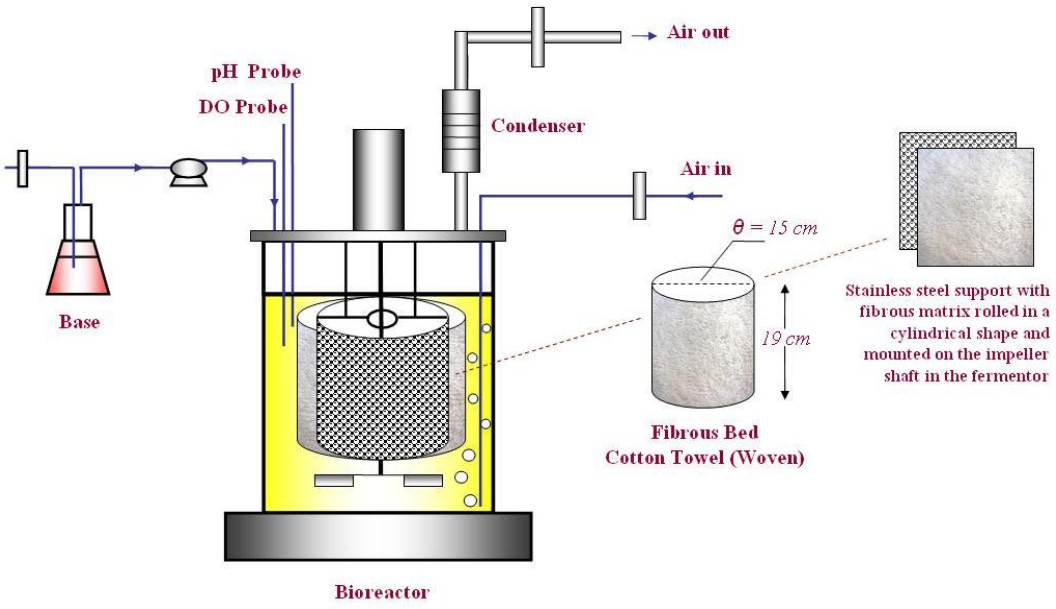


Figure 3.1 A Static bed bioreactor for filamentous fungi fermentation

3.6.2 Bioreactor start-up and cell immobilization

Before start fermentation, the bioreactor containing 3 L growth medium was autoclaved at 121°C, 15 psi for 30 min. After autoclave and cool down, the dissolved oxygen probe was calibrated with nitrogen and air. Later, 10 mL *R. oryzae* spore suspension (10^6 spores/mL) were aseptically inoculated into the bioreactor.

Spore suspension were allowed to germinate and immobilize on the fibrous matrix in growth medium. Growth phase was controlled at 30 °C for 48 h with agitation rate and agitation speed of 0.5 vvm and 700 rpm, respectively. At the end of the growth phase, the growth medium was removed from the bioreactor and the production medium was added into the bioreactor. During the fermentation phase, the bioreactor was operated at 30°C with various dissolved oxygen concentration. Besides, the pH was controlled at 6 by automatically adding 10 M NaOH. The samples were taken every 6 h until the end of the fermentation. Each batch fermentation was done when glucose depletion and lactic acid was decreased.

3.7 Analytical methods

3.7.1 Cell biomass

In shake flask culture, sample was filtered through a filter paper (whatman#4) and washed with water to remove the residues. Washed cell biomass was dried at 80 °C until constant weight.

After finished fermentation in a static bed reactor, fungal cell was removed from a perforated stainless steel, wash with water, and dried at 80 °C until constant weight.

Cell concentration was measured from the dried weight of the biomass. The cell biomass yield ($Y_{x/s}$) was calculated by the following equation:
Cell concentration

$$= \frac{\text{dry weight of immobilized of cell cotton cloth(g)} - \text{dry weight of preweighed cotton cloth (g)}}{\text{Volume of fermentation broth (L)}}$$

3.7.2 Substrate and product analyses

High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the fermentation broth. Samples from the fermentation broth was filtered through cellulose acetate membrane, and diluted with double distilled water. 15 µL diluted particle-free samples was injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm×7.8mm) maintained at 45°C in a column oven. 0.005 M H_2SO_4 was used as an eluant at 0.6 mL/min flow rate. A refractive index detector was used to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to

determine the sample concentration. The peak area was used for the comparison basis. It is noted that HPLC can detect both L-and D-lactic acids.

3.7.3 Protein determination and enzyme assay

3.7.3.1 Cell extraction

Fresh fungal mycelia was harvested by filtration and then washed thoroughly with distilled water before drying between filter papers. 1 g fungal mycelia (wet basis) was frozen at -20°C for an hour. The frozen mycelia was ground in an ice-cold mortar with 1.5 mL 50 mM Tris-HCl containing 10 mM β-mercaptoethanol pH 7.8 for 5 min. After that the ground frozen mycelia was subjected to an ultrasonic disruptor for 10 min with the working time of every 30 s and a breaking interval of 45 s. 0.5 g glass beads was added (425-600 µm). Later, the mycelial suspension was centrifuged at 12,000 g for 30 min and the supernatant was used for protein determination and enzyme assay.

3.7.3.2 Protein determination

The protein content in the cell extract sample was determined by the modified Lowry's method using a bovine serum albumin (BSA) as a standard. 0.1 mL cell extract was mixed with 3 mL reagent A, containing 2.0% sodium potassium tartrate, 1% $CuSO_4 \cdot 5H_2O$, and 2.0% Na_2CO_3 in 0.1 N NaOH. The mixture was incubated for 10 min. After incubation, 0.3 mL reagent B contained Folin phenol reagent was added into the mixture. After mixing thoroughly, the mixture was incubated for 30 min. The optical density of the mixture was measured at the wavelength of 650 nm and the protein concentration in the sample was calculated from the BSA standard curve (Appendix C).

3.7.3.3 Enzyme assay

3.7.3.3.1 NAD^+-dependent lactate dehydrogenase

NAD^+-dependent LDH activity was assayed by following the oxidation of NADH at 340 nm in the reaction mixture containing 250 µmole phosphate buffer (pH 6.5), 1 µmole NADH, 25 µmole sodium pyruvate, and 0.2 mL cell extract in the total volume of 3.15 mL. The reaction was started by addition of pyruvate (Pritchard, 1971). The reaction was started with the addition of the mycelial extract into the reaction mixture. LDH activity was detected for 7 min at 30 °C (same temperature as the culture temperature) by following the change in

absorbency at 340 nm which was later converted into μmole NADH oxidized using the calibration plot (absorbency at 340 nm versus NADH concentration in μmole) (Appendix C).

3.7.3.3.2 Alcohol dehydrogenase

ADHs activities were determined in 2 directions (an oxidation of ethanol and a reduction of acetaldehyde). In the forward reaction, the ADH activity was determined by following the oxidation of NADH at 340 nm in the reaction mixture containing 250 μmole Tris-HCl buffer pH 7.7, 250 μmole acetaldehyde, 4 μmole NADH, and 0.2 mL cell extract in the total volume of 3.15 mL (Pritchard, 1971). The reaction was started with the same manner as that described in the LDH activity assay.

In the reverse reaction, the ADH activity was measured by following the reduction of NAD^+ at 340 nm in the reaction mixture containing 250 μmole Tris-HCl buffer pH 7.7, 250 μmole ethanol, 4 μmole NAD^+, and 0.2 mL cell extract in the total volume of 3.15 mL (Pritchard, 1971). The reaction was started by adding the mycelial extract into the reaction mixture. The activity was detected from the change in absorbency at 340 nm for 7 min at 30 °C (same temperature as the culture temperature). Units of ADH(b) activity expressed as μmole NAD+ reduced per minute were determined from the calibration plot of the absorbency at 340 nm versus the concentration of NAD+ in micromole) (Appendix C).

3.7.3.3.3 Pyruvate decarboxylase

PDC activity was assayed by following NADH consumption at 340 nm. The reaction mixture contained 25 mM sodium pyruvate, 5 mM $MgCl_2$, 0.2 mM TPP (thiamine pyrophosphate), 11 U/mL ADH from baker yeast, and 0.15 mM NADH in 100 mM MES buffer pH 6.5 in a total volume of 1.2 mL (Acar et al., 2007). The reaction was started with the same action as that described in ADH and LDH activities assay.

3.7.4 Determination of apparent maximum enzyme reaction rate (ν_{max}) and substrate affinity (K_m)

Glucose, lactate, and ethanol concentrations were plotted at various fermentation times for rate determination (ν). Lactate and ethanol production rates were determined from the slope of the plot. Later, lactate and ethanol production

rates obtained from the fermentation experiments carried out with various initial glucose concentrations during the production phase were plotted against the initial glucose concentrations. The apparent maximum reaction rate for lactate and ethanol production ($\nu_{max,app}$) and the affinity of glucose substrate for lactate and ethanol ($K_{m,app}$) were described accordingly to the Michaelis – Menten Kinetics (Bailey and Oillis, 1986). Modified Lineweaver-Burk plots (1/S versus 1/ν) were used to describe $\nu_{max,app}$, $K_{m,app}$, and predict the influence of the tested inhibitors on the extrinsic affinity of substrate flux towards the metabolic routes of interest in the living *R. oryzae*. It is noted that in this modified enzyme kinetics study, glucose was used as the sole external carbon substrate for both LDH and ADH. Where $\nu_{max,app}$ represented the maximum production rate (g/L·h) and $K_{m,app}$ was defined as the initial substrate concentration (S) in g/L when the rate equaled half of $\nu_{max,app}$.

CHAPTER IV

RESULTS AND DISCUSSION

To study the improvement of lactic acid production in *Rhizopus oryzae*, various studies have used cell immobilization technique; however, none of them could not fully limit ethanol production. It is believed that limiting pyruvate flux toward undesirable pathways can be achieved by regulating enzyme in those pathways. The different types and concentration of ADH and PDC regulators were elucidated in shake flask culture of living culture *R. oryzae*. As a result of the potential regulators were found in the shake flask fermentation, they were further studied the metabolic response of *R. oryzae* in a static bed bioreactor by varying dissolved oxygen. Growth and product kinetics as well as related enzyme activities were investigated during fermentation.

4.1 In vivo regulation of alcohol dehydrogenase and lactate dehydrogenase in *R. oryzae* to improve L(+)-lactic acid fermentation

4.1.1 Morphology and metabolic responses of *R. oryzae* grown in the medium containing ADH inhibitors

Similar morphology was observed when culturing *R. oryzae* in the medium containing 1,2-diazole or 2,2,2-trifluoroethanol as compared with those grown in the simple glucose medium (control) (Fig. 4.1). Spores germinated and completely immobilized onto the cotton cloth provided. This resulted in cell-free fermentation broth. On the other hand, cell partially immobilized onto the cotton cloth when 4,4′-dithiodipyridine was present during the growth phase (Fig. 4.2). No growth was observed in the fermentation when 4-hydroxymercury benzoic acid was added into the medium (data not shown) while prolonged growth was found in the fermentation with the presence of iodoacetic acid or *N*-ethylmaleimide in the medium during the growth phase (Fig. 4.2).

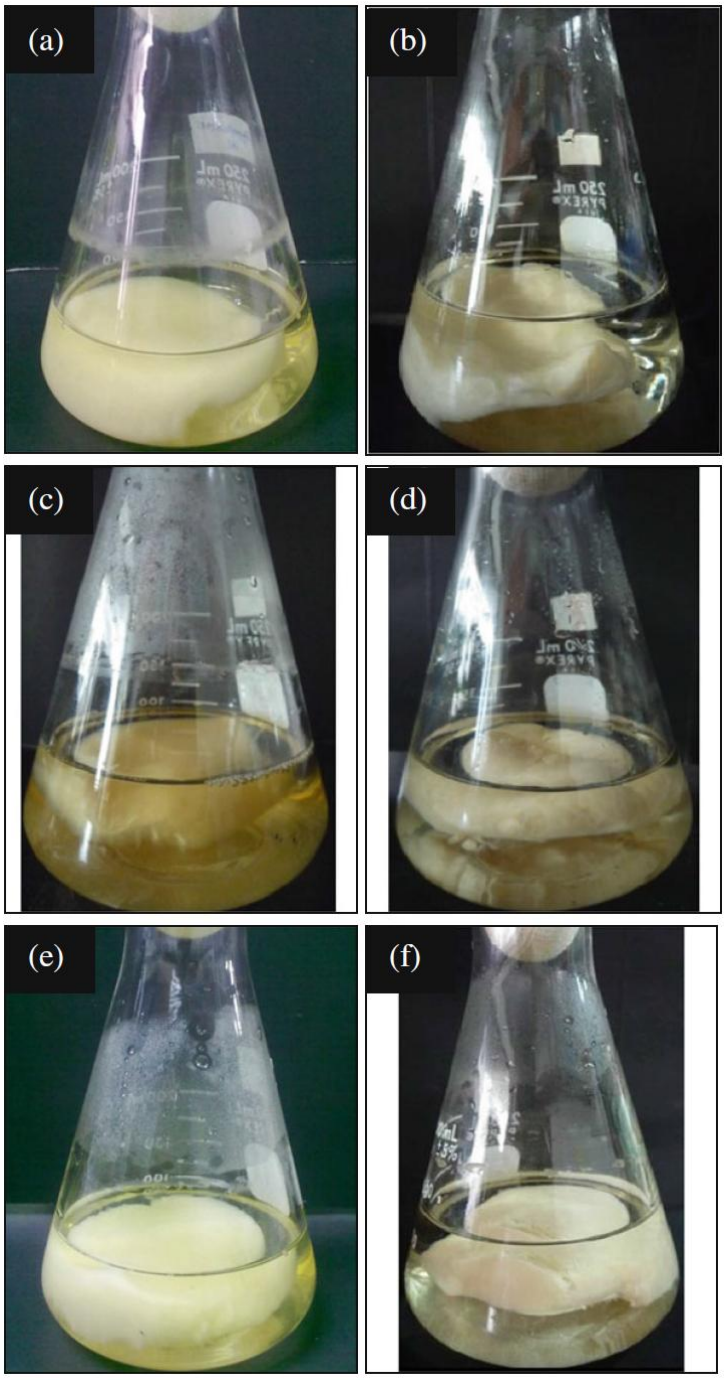


Figure 4.1 Morphology of immobilized *R. oryzae* on cotton towel grown in the medium (a) without the inhibitor at 24-h growth phase; (b) without the inhibitor at 24-h production phase; (c) containing 1 mM 1,2-diazole at 24-h growth phase; (d) containing 1 mM 1,2-diazole at 24-h production phase; (e) containing 1 mM 2,2,2-trifluoroethanol at 24-h growth phase; and (f) containing 1 mM 2,2,2-trifluoroethanol at 24-h production phase.

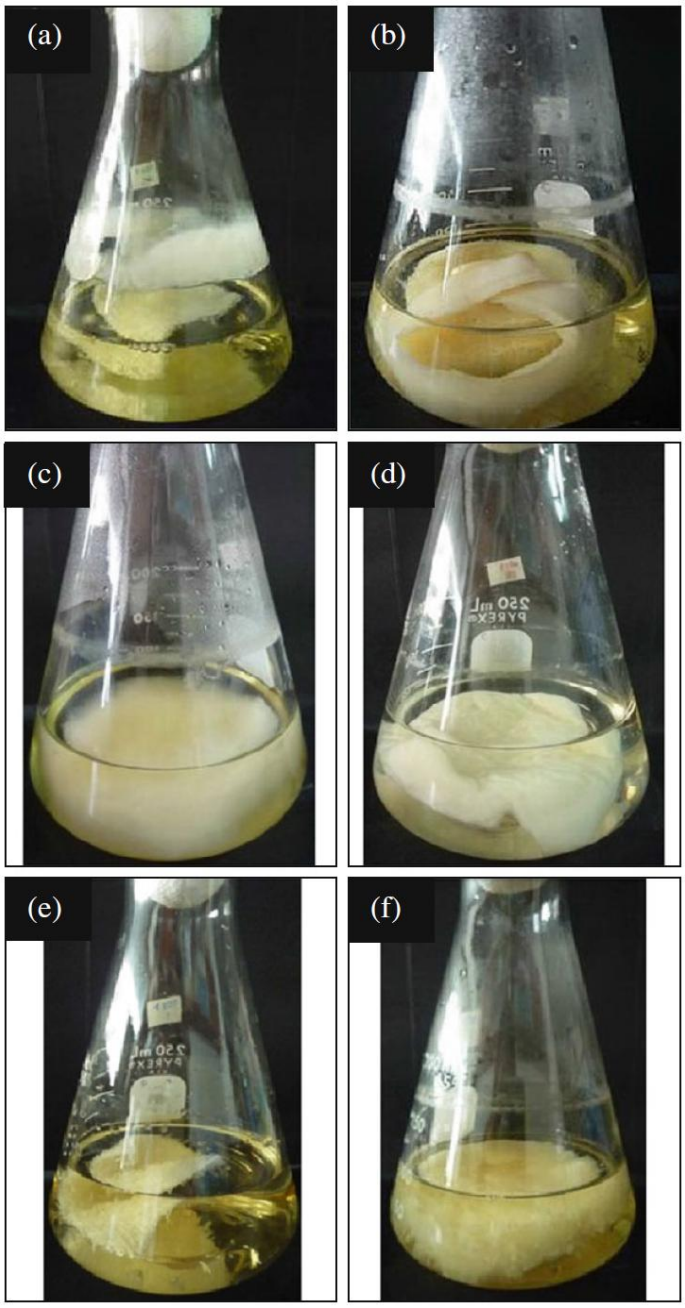


Figure 4.2 Appearance of *R. oryzae* with the presence of different ADH inhibitors (a) free cells grown in the medium containing 1 mM 4,4′-dithiodipyridine at 24-h growth phase; (b) prolonged free cell growth in the medium containing 1 mM 4,4′-dithiodipyridine at 120-h growth phase; (c) growth in the medium containing 1 M iodoacetic acid at 24-h growth phase; (d) growth in the medium containing 1 mM iodoacetic acid at 24-h production phase; (e) growth in the medium containing 1 mM

N-ethylmaleimide at 48-h growth phase; (f) prolonged growth in the medium containing 1 mM *N*-ethylmaleimide at 120-h growth phase.

Table 4.1 shows the fermentation kinetics of *R. oryzae* cultured in the glucose medium with the presence of ADH inhibitor. Similar cell, lactate, and ethanol yields were obtained from fermentations with 1,2-diazole or 2,2,2-trifluoroethanol as compared with the fermentation without inhibitor during the growth phase while more cell biomass with less lactic acid and ethanol production was obtained with the presence of 4,4′-dithiodipyridine in the growth medium. Interestingly, although ADH inhibitor, including 1,2-diazole, 2,2,2- trifluoroethanol, or 4,4′-dithiodipyradine, was initially present since the growth phase, *R. oryzae* still produced ethanol as a major product during this phase. This was perhaps due to the affinity of these inhibitors to ADH at the concentration studied could not compete with the enzyme-substrate affinity under the operating conditions during the growth phase (Singlevich and Barboriak, 1971). Moreover, yeast extract present in the growth medium for promoting spore germination and initial cell growth favored ethanol production simultaneously (Thongchul et al., 2010).

Except for the fermentations containing iodoacetic acid, *N*-ethylmaleimide, 4,4′- dithiodipyridine, or 4-hydroxymercury benzoic acid, after 48-h culture in the growth medium containing ADH inhibitor, the broth was discarded and replaced with fresh new production medium containing the inhibitor (1 mM). During the production phase, almost similar cell yields from the fermentations with 1,2-diazole or 2,2,2-trifluoroethanol to that from the fermentation without the inhibitor were obtained. It was evident that 1,2-diazole and 2,2,2-trifluoroethanol did not greatly affect the fermentation productivities as observed from the similar values to that of the fermentation without the inhibitor during the cultivation period. Only slightly increased lactate productivity was observed when 2,2,2-trifluoroethanol was present during the production phase. For the prolonged culture in the growth medium containing *N*-ethylmaleimide or 4,4′-dithiodipyridine, almost twice higher cell and ethanol yields as well as the productivities were observed. *R. oryzae* growth and product formation from fermentations with six ADH inhibitors compared with those without the inhibitor can be summarized as follows:

Cell : PCMB < IAA < NEM < CT = PZ = TFE < DSDP
Lactate : PCMB < IAA < DSDP < NEM < CT < PZ < TFE
Ethanol : PCMB < IAA < CT = PZ = TFE < NEM < DSDP

Remark: the strongest inhibiting effects of the tested ADH inhibitor towards the strongest promoting effects on cell, lactate, and ethanol production by the inhibitor are given from the minimum to the maximum (from the left to the right). Control fermentation using the simple medium is also compared with the fermentations containing different ADH inhibitors in the medium.

To prevent the negative effect of ADH inhibitors that was found particularly in the cultures containing iodoacetic acid, *N*-ethylmaleimide, or 4-hydroxymercury benzoic acid, on initial cell growth during the growth phase, *R. oryzae* was pregrown in the simple growth medium for 48 h. During the production phase, product yields and productivities correlated to the regulation of ADHs and LDH by six inhibitors were observed. Slightly increased lactate and ethanol yields compared with those of the fermentation without the inhibitor were observed in the fermentation containing 1,2-diazole or 2,2,2-trifluoroethanol while the similar cell biomass yield was obtained (Table 4.2). It was found that 1,2-diazole and 2,2,2-trifluoroethanol did not greatly influence the fermentation productivity during the production phase. On the other hand, iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid strongly repressed *R. oryzae* metabolism as observed from low product yields and productivities. The effects of 6 ADH inhibitors on pregrown *R. oryzae* metabolism during the production phase can be summarized as follows:

Cell : DSDP < NEM < PCMB < CT = PZ = TFE = IAA
Lactate : PCMB < IAA = NEM < DSDP < CT < PZ < TFE
Ethanol : IAA < DSDP < NEM < PCMB < CT < TFE < PZ

Remark: the strongest inhibiting effects of the tested ADH inhibitor towards the strongest promoting effects on cell, lactate, and ethanol production by the inhibitor are given from the minimum to the maximum (from the left to the right). Control fermentation using the simple medium is also compared along with the fermentations containing different ADH inhibitors in the medium.

Table 4.1 Product yields and productivities from fermentations by *R. oryzae* in the glucose medium with the presence of *ADH* inhibitors at 1 mM during both growth and production phases.

Inhibitors	Growth phase						Production phase					
	Product yield (g/g glucose)			Productivity (g/L•h)			Product yield (g/g glucose)			Productivity (g/L•h)		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.06±0.03	0.09±0.01	0.37±0.03	0.07±0.01	0.11±0.00	0.45±0.06	0.03±0.02	0.34±0.01	0.12±0.01	0.02±0.01	0.40±0.00	0.14±0.12
PZ(1)	0.06±0.01	0.10±0.01	0.36±0.03	0.07±0.01	0.12±0.00	0.43±0.07	0.02±0.00	0.37±0.01	0.14±0.02	0.02±0.00	0.38±0.01	0.15±0.02
TFE(1)	0.06±0.00	0.12±0.01	0.38±0.03	0.07±0.00	0.14±0.01	0.45±0.05	0.01±0.00	0.43±0.01	0.16±0.06	0.02±0.00	0.44±0.01	0.17±0.08
IAA(1)	0.01±0.00	0	0	0	0	0	0	0.01±0.00	0.06±0.00	0	0.01±0.00	0.04±0.00
PCMB(1)	0	0	0	0	0	0	0	0	0	0	0	0
NEM(1)	0	0	0	0	0	0	0.05±0.00	0.15±0.00	0.27±0.00	0.04±0.00	0.16±0.00	0.26±0.00
DSDP(1)	0.08±0.03	0.06±0.00	0.25±0.00	0.03±0.00	0	0.07±0.00	0.04±0.00	0.09±0.00	0.29±0.00	0.06±0.00	0.10±0.00	0.21±0.00

CT control, PZ 1,2-diazole, TFE 2,2,2-trifluoroethanol, PCMB 4-hydroxymercury benzoic acid, NEM N-ethylmaleimide, DSDP 4,4′-dithiodipyridine, IAA iodoacetic acid, (1) concentration of the inhibitor at 1 mM present in the medium

Strong inhibitory effects of iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid on ethanol production could be described from the irreversible binding of such inhibitors to the sulfhydryl group at the active site of ADH so that it could not serve as a ligand for the zinc atom at the catalytic site (Springman et al., 1990). Iodoacetic acid and *N*-ethylmaleimide irreversibly alkylated the S–S bond at the active site; therefore, preventing correct conformation for substrate binding (Winslow, 1981). 4,4′-dithiodipyridine also irreversibly bound with the sulfhydryl group at the active site while Hg(II) complexation with the S–S bond at the active site was found with the presence of 4-hydroxymercury benzoic acid (Zheng et al., 1997, Springman et al., 1990).

In contrast to iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid, 1,2-diazole (cofactor analog), and 2,2,2-trifluoroethanol (substrate/product analog) reversibly bound to the enzyme; therefore, the inhibition was competitive. Although 1,2-diazole and 2,2,2-trifluoroethanol did not exhibit significant reduction of ethanol formation they produced the little improvement in lactic acid yield when compared with the fermentation without the inhibitor. It is believed that ethanol production can be fully inhibited by the presence of the sufficient amounts of 1,2-diazole or 2,2,2-trifluoroethanol in the medium. As a result, improved lactic acid production can be accomplished.

Table 4.2 Product yields and productivities during the production phase with the presence of ADH inhibitors at the concentration of 1 mM of pregrown *R. oryzae* in the simple glucose medium.

Inhibitors	Production phase					
	Product yield (g/g glucose)			Productivity (g/L·h)		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.05±0.01	0.34±0.01	0.12±0.01	0.02±0.01	0.40±0.00	0.14±0.01
PZ(1[a])	0.05±0.03	0.36±0.08	0.16±0.01	0.01±0.02	0.36±0.05	0.16±0.00
TFE(1)	0.05±0.02	0.37±0.04	0.15±0.05	0.03±0.00	0.40±0.03	0.16±0.05
IAA(1)	0.05±0.00	0.02±0.00	0.02±0.00	0.01±0.00	0	0
PCMB(1)	0.04±0.00	0	0.08±0.00	0.01±0.00	0	0.01±0.00
NEM(1)	0.01±0.00	0.02±0.00	0.06±0.00	0.02±0.00	0.003±0.00	0.02±0.00
DSDP(1)	0	0.11±0.00	0.04±0.00	0.01±0.00	0.12±0.00	0.01±0.00

In the growth phase, *R. oryzae* was pregrown in the simple growth medium containing 50 g/L glucose and 5 g/L yeast extract for 48 h at 30 °C and 200 rpm.
CT control, PZ 1,2-diazole, TFE 2,2,2-trifluoroethanol, PCMB 4-hydroxymercury benzoic acid, NEM *N*-ethylmaleimide, DSDP 4,4′-dithiodipyridine, IAA iodoacetic acid, (1[a]) concentration of the inhibitor at 1 mM present in the medium.

4.1.2 1,2-diazole and 2,2,2-trifluoroethanol as the competitive ADH inhibitors

Competitive inhibition by 1,2-diazole or 2,2,2-trifluoroethanol was observed in the fermentations with different concentrations (0.01 mM – 10 mM) of these 2 inhibitors. During the growth phase, it was found that varied concentrations of 1,2-diazole did not significantly influence the fungal metabolism as observed from the similar yields of cell biomass (~0.04-0.07 g/g glucose), lactate (~0.08-0.12 g/g glucose), and ethanol (~0.36-0.42 g/g glucose) (Table 4.3). But increasing lactic acid

yield with decreasing ethanol yield was observed during the production phase when increasing 1,2-diazole concentration from 0 mM to 0.1 mM and 2,2,2-trifluoroethanol concentration from 0 mM to 1 mM. During the production phase, the highest lactic acid yield of 0.44 g/g glucose was observed from the fermentation with 0.1 mM 1,2-diazole. Along the cultivation, almost similar cell biomass, lactate, and ethanol productivities were found in the fermentations with 1,2-diazole and 2,2,2-trifluoroethanol at different concentrations when compared with those in the fermentation without the inhibitor (Table 4.3).

The effect of 1,2-diazole and particularly 2,2,2-trifluoroethanol on lactate yield was more evident when they were added during the production phase of pregrown *R. oryzae* (Table 4.4). The highest lactate yield of 0.47 g/g glucose was found at the lowest 2,2,2-trifluoroethanol concentration of 0.01 mM used in this work which was about 38% increasing yield compared with that of the control fermentation (0.34 g/g glucose). Increasing 1,2-diazole concentration in the production medium slightly influenced product yields. Nevertheless, ethanol (~0.12-0.19 g/g glucose) and cell (~0.04-0.05 g/g glucose) yields from the fermentations with 1,2-diazole or 2,2,2-trifluoroethanol were not lower than those from the simple glucose fermentation as expected. It was found that productivities during the production phase were slightly affected by varying the concentration of *ADH* inhibitor (Table 4.4). The highest lactate productivity of 0.43 g/L·h was achieved when 0.01 mM 2,2,2-trifluoroethanol was present in the production medium.

It was expected that lower ethanol yield while increasing lactic acid yield should have been achieved from fermentations when 1,2-diazole or 2,2,2-trifluroethanol were present; however, the kinetics data revealed that the in vivo regulation of lactic acid and ethanol production by these 2 inhibitors during cultivation of immobilized *R. oryzae* was not clearly seen. It could be explained that the presence of these 2 inhibitors in the living *R. oryzae* culture with complex enzyme system caused metabolic shift not only at the targeted alcohol fermentation pathway as previously expected. Better understanding in metabolic flux balance when these two inhibitors are present in the living *R. oryzae* culture is required in order to achieve limited ethanol production and enhanced lactic acid production.

Table 4.3 Product yields and productivities from fermentations by *R. oryzae* in the glucose medium containing 1,2-diazole or 2,2,2-trifluoroethanol at different concentrations during both growth and production phases.

Inhibitors	Growth phase						Production phase					
	Product yield (g/g glucose)			Productivity (g/L·h)			Product yield (g/g glucose)			Productivity (g/L·h)		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.06±0.03	0.09±0.01	0.37±0.03	0.07±0.01	0.11±0.00	0.45±0.06	0.03±0.02	0.34±0.01	0.12±0.01	0.02±0.01	0.40±0.00	0.14±0.12
PZ(0.01*)	0.07±0.00	0.11±0.01	0.42±0.07	0.07±0.01	0.13±0.03	0.46±0.03	0.08±0.05	0.36±0.15	0.13±0.04	0.04±0.02	0.33±0.15	0.12±0.04
TFE(0.01)	0.04±0.04	0.09±0.03	0.39±0.03	0.07±0.01	0.10±0.04	0.41±0.00	0.06±0.07	0.32±0.14	0.13±0.02	0.01±0.00	0.36±0.11	0.15±0.00
PZ(0.1)	0.05±0.01	0.11±0.01	0.40±0.05	0.07±0.01	0.13±0.02	0.49±0.08	0.02±0.01	0.44±0.10	0.18±0.04	0.03±0.00	0.44±0.07	0.18±0.02
TFE(0.1)	0.06±0.00	0.09±0.02	0.40±0.03	0.07±0.00	0.12±0.02	0.49±0.01	0.04±0.03	0.39±0.02	0.17±0.01	0.04±0.04	0.43±0.09	0.18±0.00
PZ(1)	0.06±0.01	0.10±0.00	0.36±0.03	0.07±0.01	0.12±0.00	0.43±0.07	0.02±0.00	0.37±0.01	0.14±0.02	0.02±0.00	0.38±0.01	0.15±0.02
TFE(1)	0.06±0.00	0.12±0.01	0.38±0.03	0.07±0.00	0.14±0.01	0.45±0.05	0.01±0.00	0.43±0.01	0.16±0.06	0.02±0.00	0.44±0.01	0.17±0.08
PZ(10)	0.05±0.00	0.11±0.02	0.41±0.02	0.06±0.01	0.12±0.02	0.49±0.06	0.01±0.01	0.32±0.07	0.19±0.01	0.01±0.01	0.33±0.05	0.19±0.00
TFE(10)	0.06±0.01	0.08±0.01	0.39±0.10	0.06±0.01	0.09±0.03	0.42±0.02	0.02±0.02	0.33±0.03	0.16±0.03	0.02±0.02	0.29±0.02	0.14±0.05

Remarks: CT = control; PZ = 1,2-diazole; TFE = 2,2,2-trifluoroethanol; (*) = concentration of the inhibitor in mM present in the medium.

Table 4.4 Product yields and productivities during the production phase with the presence of various concentrations of 1,2-diazole or 2,2,2-trifluoroethanol of pregrown *R. oryzae* in the simple glucose medium.

Inhibitors	Production phase					
	Product yield (g/g glucose)			Productivity (g/L·h)		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.05±0.01	0.34±0.01	0.12±0.01	0.02±0.01	0.40±0.00	0.14±0.01
PZ(0.01[a])	0.04±0.02	0.37±0.08	0.14±0.07	0.01±0.01	0.37±0.06	0.15±0.08
TFE(0.01)	0.04±0.02	0.47±0.03	0.15±0.03	0.02±0.01	0.43±0.00	0.14±0.02
PZ(0.1)	0.04±0.03	0.38±0.04	0.16±0.01	0.01±0.01	0.37±0.06	0.16±0.01
TFE(0.1)	0.04±0.03	0.42±0.01	0.19±0.03	0.04±0.03	0.42±0.04	0.19±0.02
PZ(1)	0.05±0.03	0.36±0.08	0.16±0.01	0.01±0.02	0.36±0.05	0.16±0.00
TFE(1)	0.05±0.02	0.37±0.04	0.15±0.05	0.03±0.00	0.40±0.03	0.16±0.05
PZ(10)	0.04±0.04	0.34±0.01	0.19±0.06	0.07±0.06	0.35±0.00	0.19±0.05
TFE(10)	0.04±0.04	0.32±0.00	0.13±0.01	0.02±0.00	0.34±0.02	0.13±0.01

- In the growth phase, *R. oryzae* was pregrown in the simple growth medium containing 50 g/L glucose and 5 g/L yeast extract for 48 h at 30 °C and 200 rpm
- CT control, PZ 1,2-diazole, TFE 2,2,2-trifluoroethanol
- [a]Concentration of the inhibitor in millimolars present in the medium

4.1.3 In vivo effects of ADH inhibitors on enzyme activities during lactic acid fermentation

Figure 4.3 represents changes in LDH and ADH activities throughout the cultivation period when 1,2-diazole or 2,2,2-trifluoroethanol were present in both growth and production phases. During the growth phase (0–48 h), enzyme activities increased with the increasing time. The enzyme activities continuously dropped during the production phase (48–120 h). The highest activities were found at 48-h cultivation. Similar results were investigated by Chotisubha-anandha and coworkers (Chotisubha-anandha et al., 2011). They observed the increase in the activity of LDH with the increasing fermentation time (from 0 to 48 h). Later, the activity dropped due to glucose depletion. When 2,2,2-trifluoroethanol was present, the activities of LDH, ADH(f), and ADH(b) were dramatically increased while similar activities were found in the fermentation with 1,2-diazole when compared with those in the fermentation in the simple glucose medium. The increasing enzyme activities when 2,2,2-trifluoroethanol was added into the culture is presumably explained by the analogous chemical structures of 2,2,2- trifluoroethanol and ethanol. This might lead to the increasing enzyme expression level due to cellular response to the substrate/product analog. In case of 1,2-diazole, it competes with the cofactors NAD+/NADH; thus, it does not directly influence the enzyme expression in the living *R. oryzae* culture. Low enzyme activities were found in the fermentations containing iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, and 4-hydroxymercury benzoic acid along the cultivation period (data not shown).

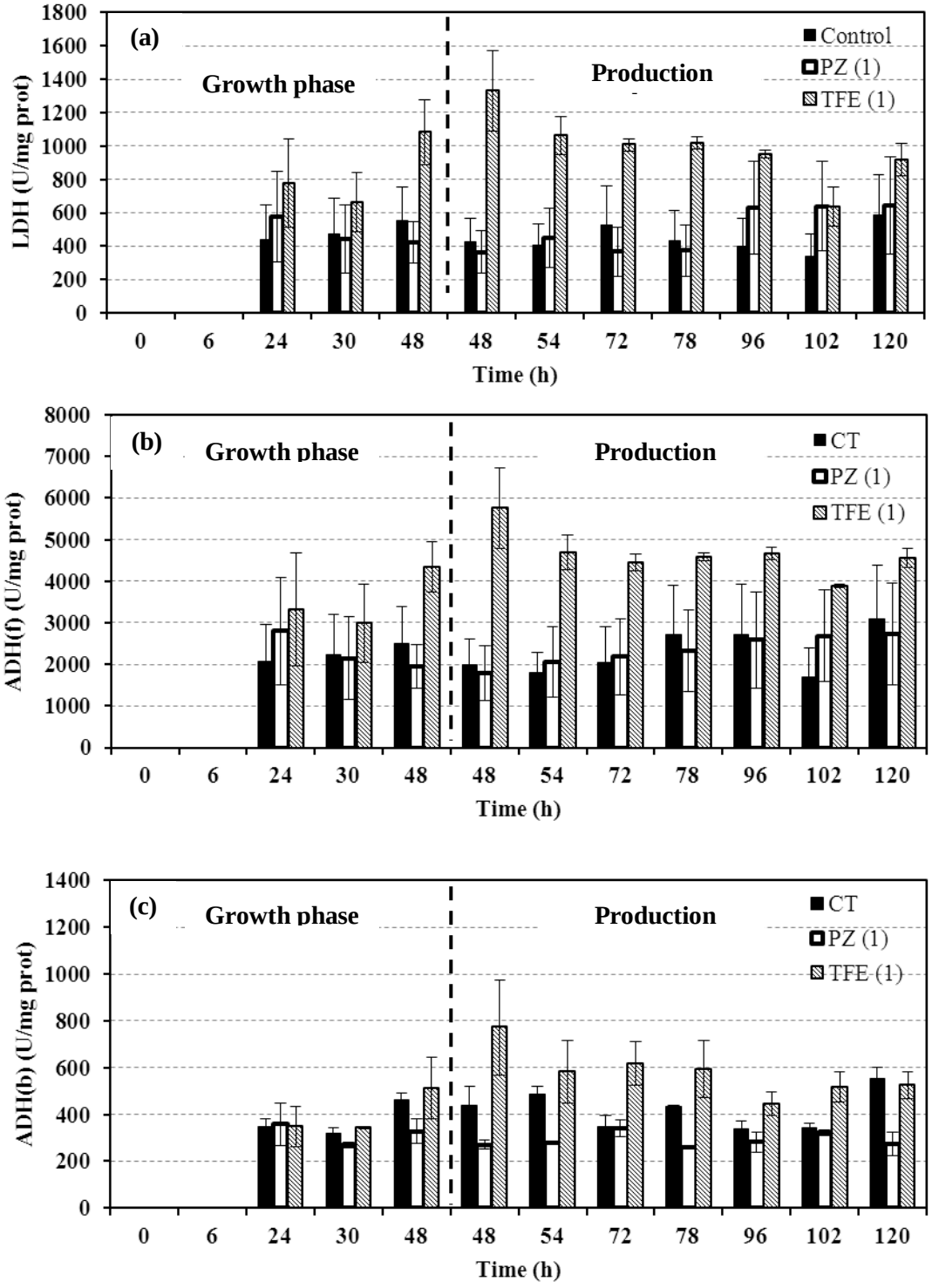


Figure 4.3 Enzyme activities during the fermentation by *R. oryzae* in the glucose medium containing 1,2-diazole (PZ) or 2,2,2-trifluoroethanol (TFE) at 1 mM; (a) LDH; (b) ADH(f)—acetaldehyde to ethanol; and (c) ADH(b)—ethanol to acetaldehyde.

Similar trends of changes in enzyme activities were found in the fermentations of pregrown *R. oryzae* compared with those fermentations when the inhibitors were added during the growth phase and that without the inhibitor. Firstly, *R. oryzae* was pregrown in the simple glucose medium during the growth phase. Later in the production phase the inhibitor was added into the medium. Figure 4.4 shows the enzyme activities at 48 h cultivation. No enzyme activity was detected when iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid were added into the production medium. While the fermentation with 2,2,2-trifluoroethanol led to increasing LDH and ADHs activities. The presence of 1,2-diazole in the medium did not seem to affect the enzyme expression (Fig. 4.4). As stated in the previous studies, the binding of iodoacetic acid, *N*-ethylmaleimide, 4,4′-dithiodipyridine, or 4-hydroxymercury benzoic acid to the enzyme active site are irreversible (Taber, 1998; Zheng et al., 1997; and Yoneya and Sato, 1979). Therefore, the presence of these four inhibitors during cultivation of living *R. oryzae* prevented further enzyme-substrate binding. This eventually led to no enzyme activity found when performing the enzyme assays of the mycelial extract later.

It is obvious that the addition of the inhibitor at an early stage of the fermentation caused metabolic repression (Figs. 4.4 and 4.5). When the inhibitor was added only during the production phase, higher enzyme expression levels were found compared with those with the inhibitors since the growth phase (Figs. 4.4 and 4.5). Almost 50 times increased LDH activity was observed during the production phase with the presence of 2,2,2-trifluoroethanol of *R. oryzae* culture that was pregrown in the simple glucose medium. Although ADH(f) activity was lower than LDH activity in the fermentations containing the inhibitors, it was still higher than that found in the control fermentation. The increasing enzyme activity when the inhibitor was present only in the production phase suggested that the addition of ADH inhibitor during the production phase is sufficient for improving lactic acid production. Also, the chance of cell damage by adding the inhibitor only in the production phase can be prevented.

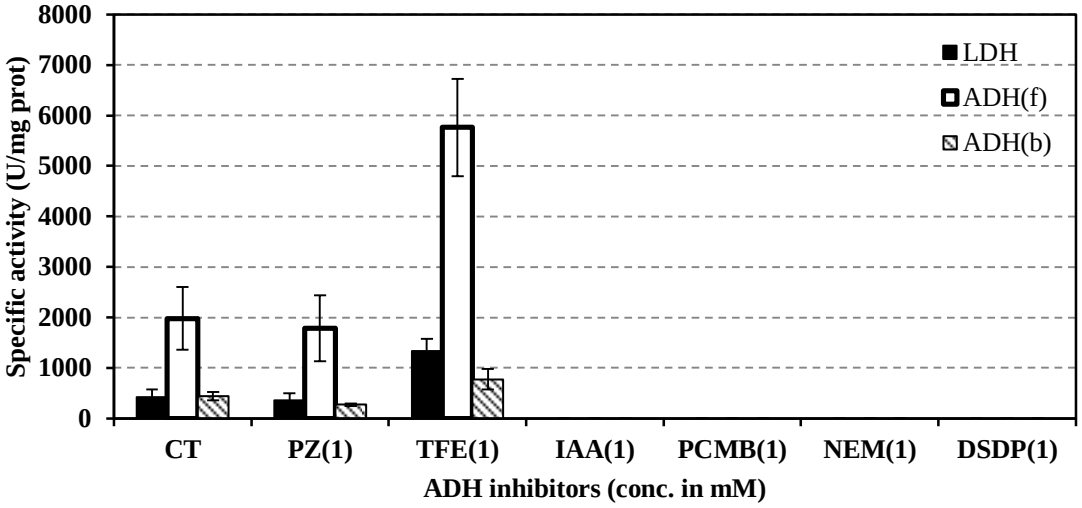


Figure 4.4 Activities of LDH and both ADH(f) and ADH(b) at 48 h. During growth and production phases, *R. oryzae* was cultivated in the medium containing various ADH inhibitors at 1 mM.

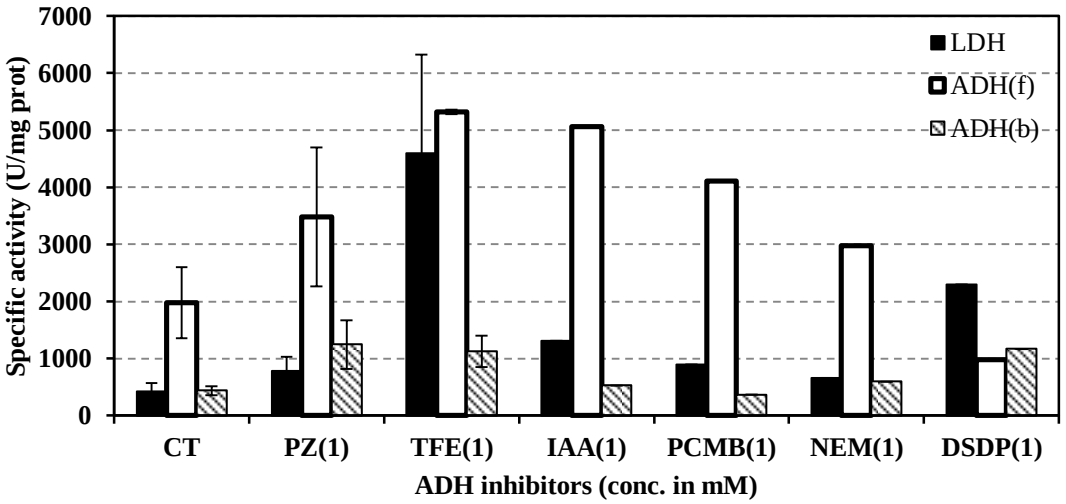


Figure 4.5 Activities of LDH and both ADH(f) and ADH(b) at 48 h with various ADH inhibitors at 1 mM. During the growth phase, *R. oryzae* was pregrown at the simple glucose medium at 30 °C, 200 rpm.

In this study, the inhibition of ADH by the inhibitors associated with the lower ADH activity and eventually led to less ethanol produced during the cultivation of *R. oryzae* was expected. But in the living cells, the addition of the potential ADH inhibitors led to highly increasing specific enzyme activities of LDH and ADHs compared with the specific activities observed in the glucose fermentation without the inhibitor (control). These results are presumably explained by the influence of the inhibitor added as well as the effects of other environmental factors such as local nutrient/oxygen transport. For example, for all cases (with or without the inhibitors) at some local area likely at the central core of the immobilized cell matrix, oxygen might be limited; thus, *R. oryzae* cells in such an area readily entered alcohol fermentative pathway. With the presence of the inhibitor, some ADHs produced were bound with the inhibitor reversibly or irreversibly depending on the type of inhibition. Presumably, cells responded to this phenomenon by increasing ADH expression. As a consequence, largely increased ADH activities were observed as compared with those in the control cultivation. Increasing LDH specific activity was also observed. This might be explained by the metabolic shift at pyruvate branch point when the ADH inhibitor was present because ADH inhibitors controlled flows through all the metabolic routes. The concentrations of these inhibitors were the regulating signals which governed the activities of the controlling enzymes at the pyruvate branch point.

4.2 1, 2 – diazole and 2, 2, 2 – trifluoroethanol and their regulatory effects on ethanol and lactic acid formation in the living culture of *R. oryzae*

4.2.1 Fungal growth and morphology of immobilized *R. oryzae* in the glucose medium

Spore germination and preculture of *R. oryzae* in the simple growth medium containing 50 g/L glucose was initiated for fermenting L – lactic acid later during the production phase. After inoculating for 6 h, spores germinated and fully immobilized onto the cotton cloth provided resulting in the cell – free fermentation broth. Similar cell growth with the final cell concentration of 3.81 ± 0.09 g/L from all preculture experiments was obtained. After 48 h, the growth medium was removed and the preculture was transferred into the production medium to observe the regulatory effects of 2 selected ADH inhibitors (1, 2 – diazole and 2, 2, 2 – trifluoroethanol) on the fungal growth and the glucose uptake rate for lactate and ethanol formation to describe the activities of both enzymes extrinsically in the living *R. oryzae* (Thitiprasert et al., 2011). During the production phase, immobilized *R.*

oryzae grew slowly as observed from the slightly increase in cell dry weight of approximately 0.35 – 1.18 g/L. From the central metabolic pathway of *R. oryzae* shown in Fig. 4.6, it is believed that the slow growth could be the consequence of the limited nitrogen source in the production medium which promoted most of glucose flux driven toward pyruvate via glycolysis for either lactate or ethanol production (Longacre et al., 1997).

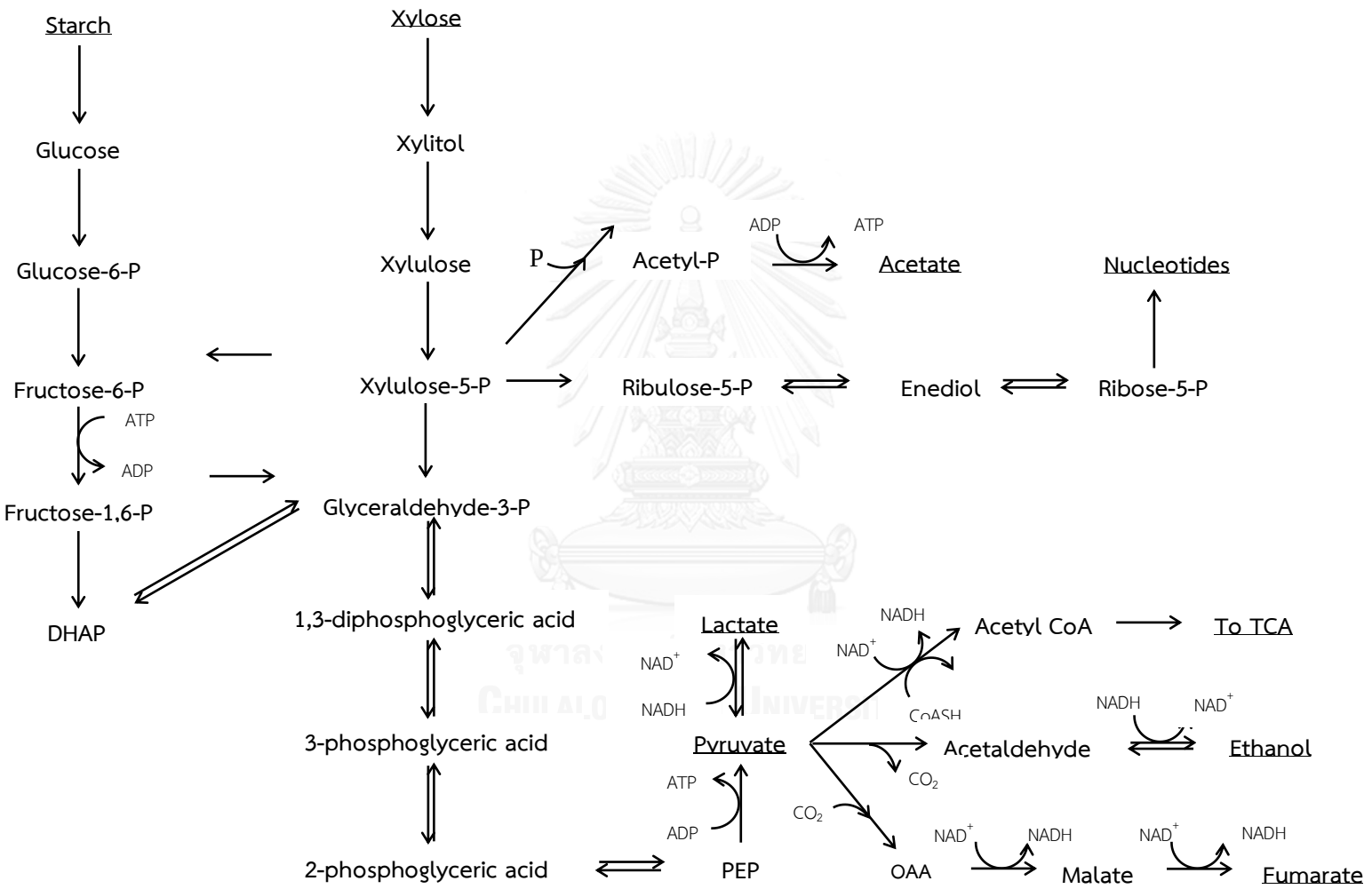


Figure 4.6 Central metabolic pathway of *R. oryzae* (Thongchul, 2013).

4.2.2 Metabolic response of living R. oryzae grown in different glucose concentrations with ADH inhibitors

Table 4.5 shows the glucose uptake for L – lactate and ethanol production of the pregrown *R. oryzae* in the production medium containing various initial glucose concentrations. Glucose uptake rate was increased with the increasing initial glucose concentration regardless of the presence of the enzyme inhibitors. It was found that at the higher initial glucose concentration than 50 g/L, the trend of the glucose uptake rate in the control culture (without ADH inhibitors) kept increasing. In the cultures containing ADH inhibitors, on the other hand, the rates at the same initial glucose concentration seemed slightly higher than that of the control culture before starting to drop when the concentration reached 90 g/L. It is evident that high glucose concentration can be used in the typical fermentation of *R. oryzae* (control culture) while the fermentation with ADH inhibitors especially 2, 2, 2 – trifluoroethanol somehow limited the glucose uptake at high initial glucose concentration. From this finding, it was implied that to enhance lactate production by limiting ethanol production pathway, not only adding the ADH inhibitors into the culture medium, the fermentation should have been done at slightly low initial glucose concentration in order to accelerate the glucose flux toward the desirable metabolic route (Umulis et al., 2005).

Increasing initial glucose concentration in the production medium of the control culture did not affect lactate yield but rather improved its productivity. The increase in lactate productivity with the increasing initial glucose concentration was also found in the cultures grown in the production medium containing either 1, 2 – diazole or 2, 2, 2 – trifluoroethanol. Similarly to what observed in glucose uptake, lactate production in the control culture was not limited by the initial glucose concentration as clearly seen from the increasing production rate from 10 – 90 g/L initial glucose concentration studied. While the fermentation with the inhibitors somewhat resulted in the increasing lactate productivity when increasing the initial glucose fermentation; however, the rate was lower than that obtained in the control culture grown at the same initial glucose concentration. Lactate yield was supposed to increase when ADH inhibitors were present during the fermentation of living *R. oryzae* as the consequence of more pyruvate flux driven toward lactate; however, the yields rather remained unchanged in all tests reported in this study (Longacre et al., 1997).

Table 4.5 Product yields and productivities during the production phase with the presence of ADH inhibitors at the concentration of 1 mM of pregrown *R. oryzae* in the simple glucose medium containing various initial glucose concentration.

Initial glucose concentration (g/L)	10	20	30	50	70	90
Glucose uptake rate (g/L•h)						
CT	0.36±0.07	0.57±0.08	0.74±0.12	1.12±0.25	1.15±0.13	1.52±0.22
PZ (1)	0.31±0.00	0.61±0.02	0.68±0.17	1.09±0.28	1.58±0.32	1.54±0.39
TFE (1)	0.35±0.05	0.59±0.05	0.75±0.21	0.92±0.11	1.65±0.00	0.82±0.26
Lactate yield (g/g)						
CT	0.32±0.00	0.34±0.10	0.28±0.07	0.30±0.00	0.25±0.06	0.34±0.03
PZ (1)	0.56±0.03	0.34±0.10	0.27±0.07	0.31±0.04	0.28±0.08	0.21±0.01
TFE (1)	0.35±0.14	0.35±0.05	0.20±0.04	0.33±0.01	0.21±0.04	0.45±0.03
Lactate production rate (g/L•h)						
CT	0.12±0.02	0.21±0.08	0.28±0.07	0.34±0.07	0.29±0.04	0.51±0.12
PZ (1)	0.18±0.01	0.21±0.07	0.26±0.08	0.39±0.12	0.44±0.03	0.36±0.06
TFE (1)	0.13±0.06	0.21±0.05	0.20±0.06	0.31±0.04	0.34±0.06	0.38±0.15
Ethanol yield (g/g)						
CT	0.32±0.00	0.30±0.03	0.19±0.03	0.12±0.02	0.06±0.00	0.10±0.00
PZ (1)	0.49±0.05	0.27±0.00	0.41±0.10	0.12±0.00	0.09±0.02	0.11±0.00
TFE (1)	0.62±0.00	0.27±0.04	0.21±0.00	0.16±0.07	0.25±0.02	0.25±0.04
Ethanol productivity (g/L•h)						
CT	0.13±0.00	0.17±0.01	0.14±0.00	0.13±0.05	0.06±0.00	0.17±0.00
PZ (1)	0.16±0.02	0.17±0.00	0.27±0.00	0.15±0.00	0.14±0.05	0.20±0.00
TFE (1)	0.21±0.03	0.16±0.01	0.19±0.00	0.14±0.05	0.41±0.03	0.20±0.04

Remarks: CT control; PZ (1) 1, 2 – diazole at 1 mM; TFE (1) 2, 2, 2 – trifluoroethanol at 1 mM.

Contrary to lactate production, lower ethanol yield was obtained when the initial glucose concentration in the production medium was increased in all 3 cases studied (control culture, cultures with 1, 2 – diazole and 2, 2, 2 – trifluoroethanol). This strongly confirmed that high glucose concentration is preferable to glucose uptake for lactate production in the living *R. oryzae* although glucose uptake rate might be slightly low. This implies the possibility of fed – batch or repeated batch cultures of immobilized *R. oryzae* for high lactate production rate at a slightly high initial glucose concentration with the limited ethanol production yield. Instead of decreasing ethanol yield when adding ADH inhibitors into the production medium, living *R. oryzae* consumed more glucose for ethanol when compared with those in the control cultures without the inhibitor. Contrary to glucose uptake and lactate production, increasing initial glucose concentration did not affect ethanol productivity in the cultures either with or without the presence of inhibitor.

The evidences of slow growth observed during the production phase, higher rates of glucose uptake and lactate formation with the unchanged lactate yield but unchanged ethanol production rate with a decreasing ethanol yield confirmed that the glucose flux was driven towards pyruvate with the limited shunt to cell biomass production (Longacre et al., 1997). Living *R. oryzae* possibly attempted to consume glucose for lactate when ADH inhibitor was present in the production medium. Instead of completing TCA/ETC coupling for ATP and NAD^+/NADH generation, some extent of ethanol was produced for cofactor regeneration which was required in lactic acid production (Fig.4.6). The fluctuation in ethanol yield observed in the cultures with the presence of the inhibitors could have also indicated the reversible reactions of acetaldehyde and ethanol for regenerating NAD^+ and NADH in the fermentative alcoholic pathway of living *R. oryzae* (Dolega, 2010 and Ma et al., 2012).

4.2.3 Regulation of 1, 2 – diazole and 2, 2, 2 – trifluoroethanol in the culture of living R. oryzae

The fermentation kinetic profiles observed when culturing *R. oryzae* for L – lactate production with the presence of ADH inhibitors are not significantly different when compared with that in the control culture grown in the glucose production medium (Figs. 4.7 – 4.9). The findings in this study were consistent with those reported in Thitiprasert et al. (2011) that 1, 2 – diazole and 2, 2, 2 –

trifluoroethanol rather enhanced both lactate and ethanol production in the living *R. oryzae* (Bailey and Ollis, 1986).

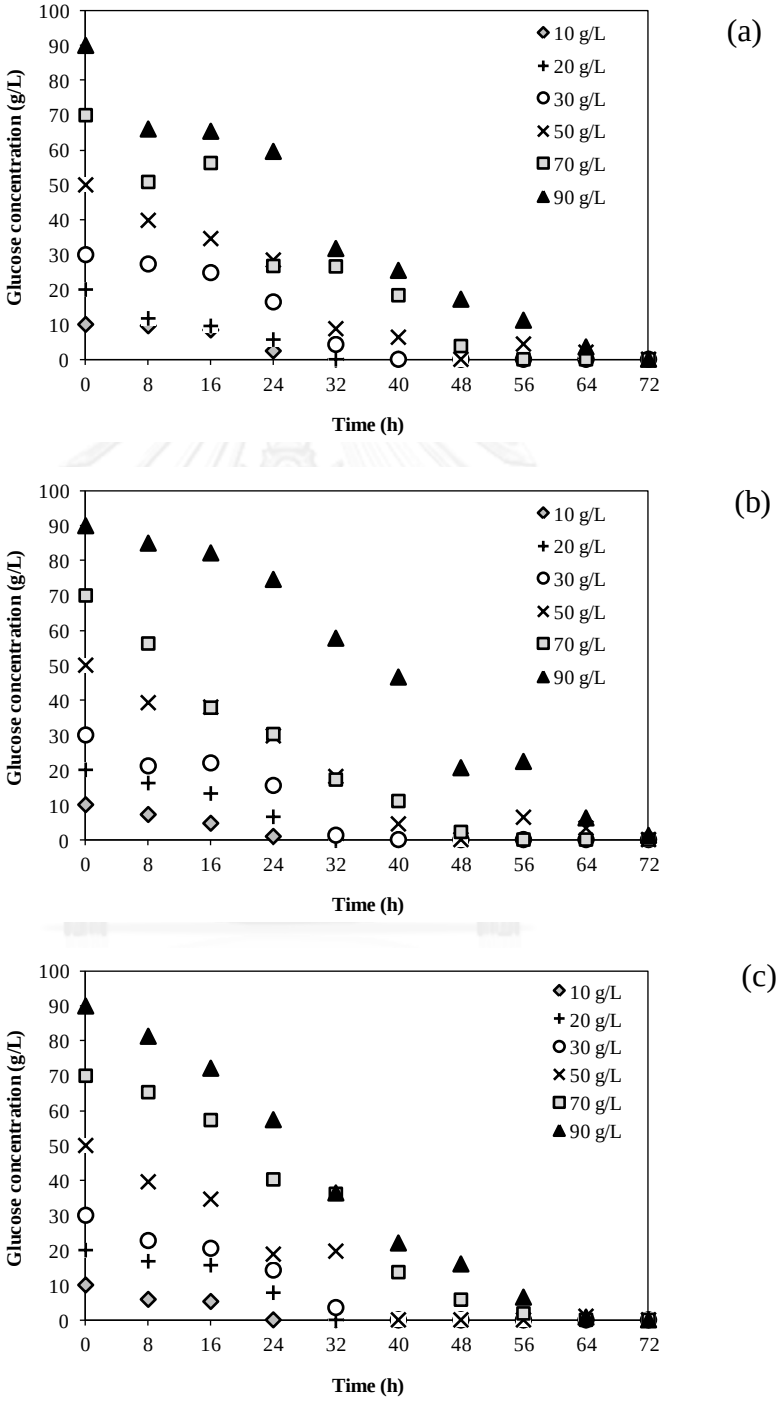


Figure 4.7 Glucose consumption during the fermentation of immobilized *R. oryzae* grown in the production medium at different initial glucose concentration (10 – 90 g/L), with 1 mM 1, 2 – diazole (a), with 1 mM 2, 2, 2 – trifluoroethanol (b), and without any enzyme inhibitor (control) (c).

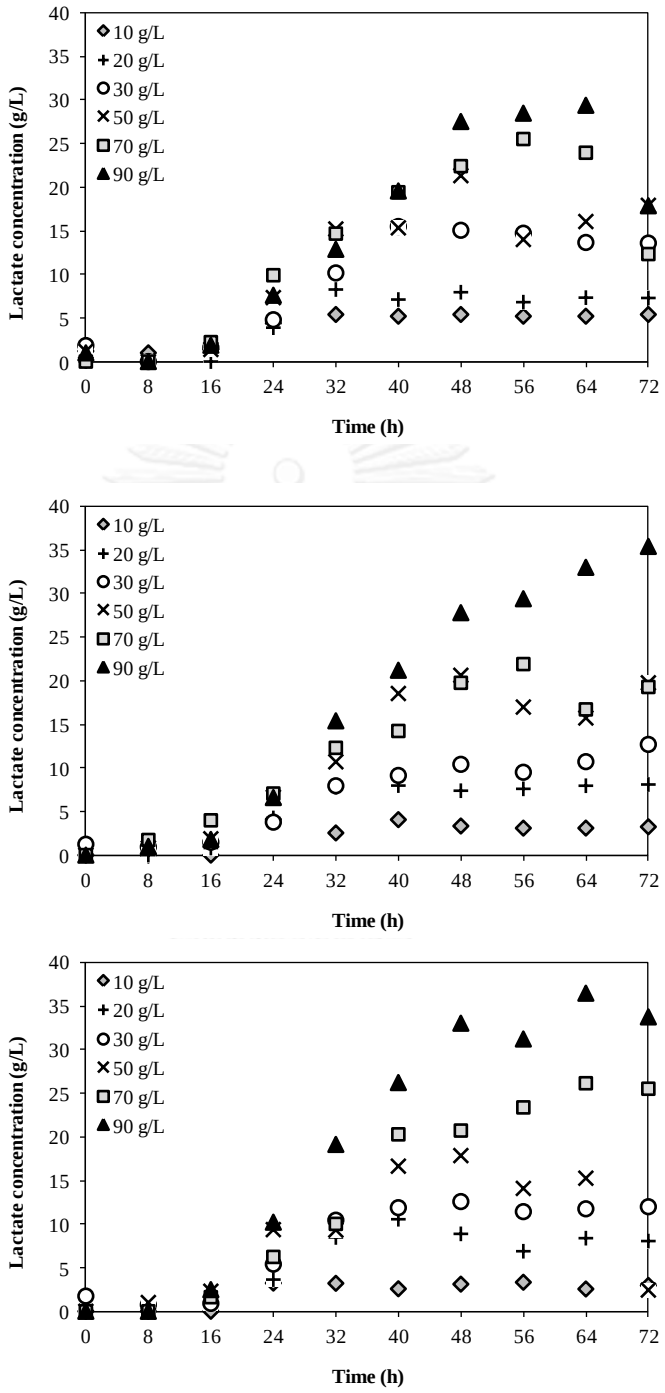


Figure 4.8 L – lactic acid production during the fermentation of immobilized *R. oryzae* grown in the production medium at different initial glucose concentration (10 – 90 g/L), with 1 mM 1, 2 – diazole (a), with 1 mM 2, 2, 2 – trifluoroethanol (b), and without any enzyme inhibitor (control) (c).

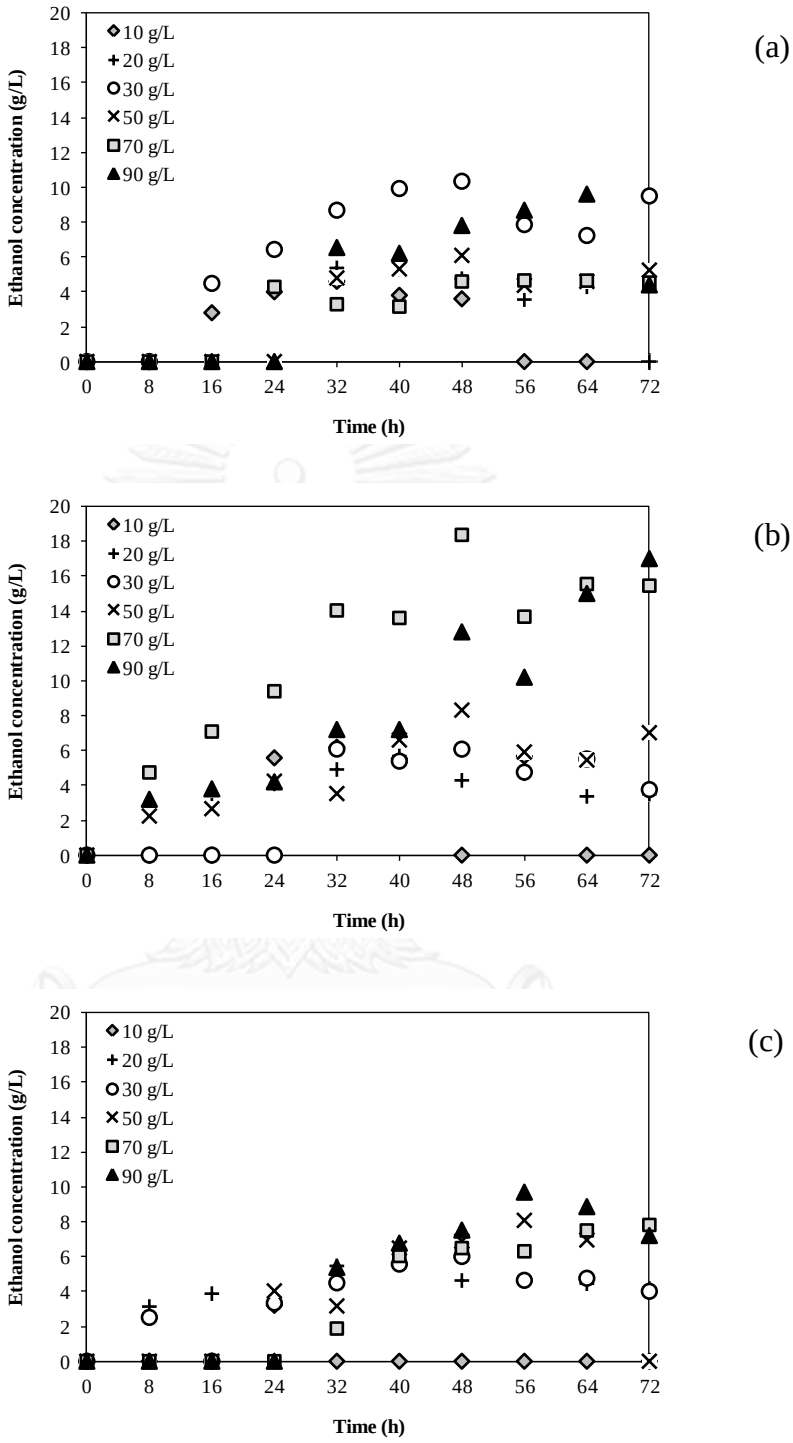


Figure 4.9 Ethanol production during the fermentation of immobilized *R. oryzae* grown in the production medium at different initial glucose concentration (10 – 90 g/L), with 1 mM 1, 2 – diazole (a), with 1 mM 2, 2, 2 – trifluoroethanol (b), and without any enzyme inhibitor (control) (c).

Many in vitro activity tests have been reported that 1, 2 – diazole and 2, 2, 2 – trifluoroethanol competitively bound at the coenzyme binding site and the catalytic site of ADH, respectively (Figs.4.10 and 4.11).

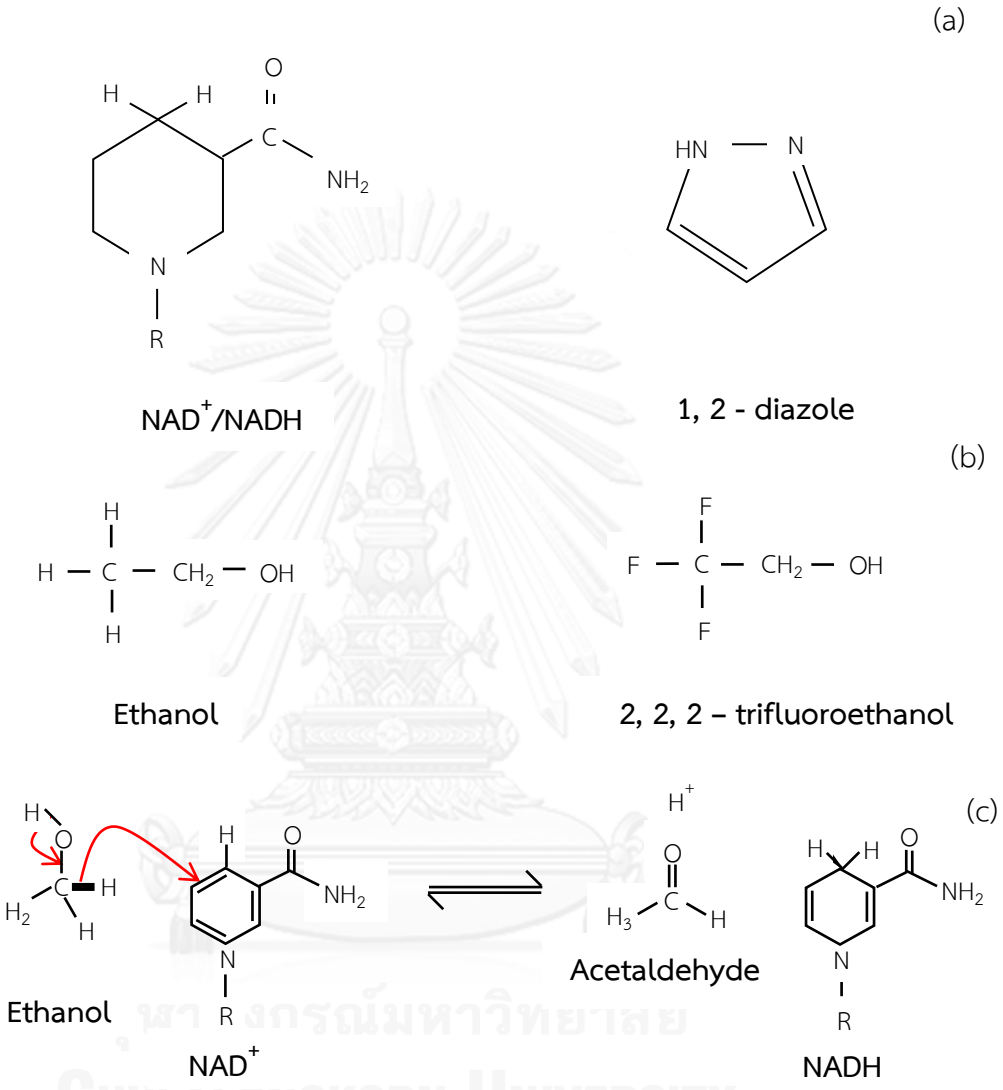
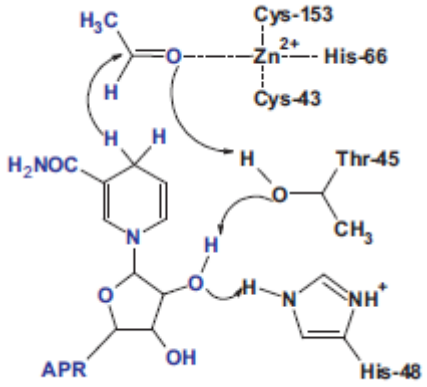
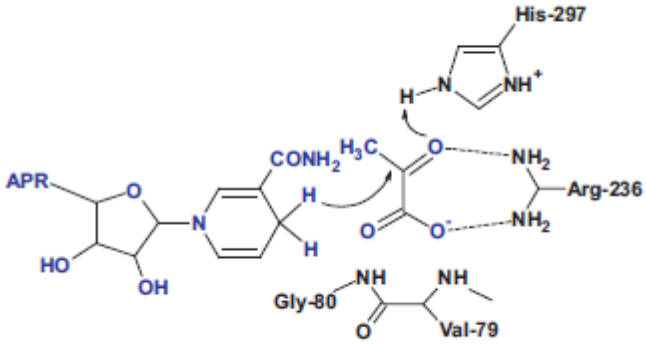


Figure 4.10 Structure of alcohol dehydrogenase (ADH) substrates and their analogs, cofactor (NAD^+/NADH) that binds at the coenzyme binding domain and its analog (1, 2 – diazole) (a) and substrate (ethanol) that binds at the catalytic domain and its analog (2, 2, 2 – trifluoroethanol (b) and its reversible redox mechanism (c).

(a)



(b)



(c)

APR structure

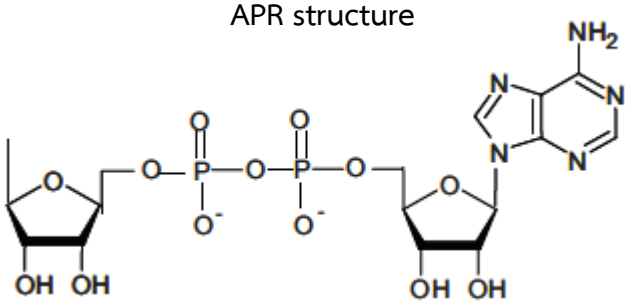


Figure 4.11 Key oxidoreductases found in *R. oryzae* metabolism and their proximity reactions which include ADH catalyzes the reduction of acetaldehyde to ethanol (a) and LDH reduces pyruvate to lactate (b). Arrows indicate the movements of electron pairs in the catalytic reactions. APR represents structural backbone of NAD^+/NADH (Ma et al., 2012).

Though it was claimed that substrate/cofactor analogs provided a great impact in increasing the yield and productivity of the desired product while minimizing byproduct formation, those were in vitro determined using the crude extract of intact enzymes of interest (Taber, 1998; Goldstein et al., 1971; and Singlevich and Barboriak, 1971). On the other hand, applying the inhibitors directly into the living culture resulted in the slightly different results compared to those in the in vitro test. It should be noted that adding 1, 2 – diazole and 2, 2, 2 – trifluoroethanol did not manipulate only a single enzymatic reaction in the living system but it also rather affected the whole metabolic response of *R. oryzae* (Umulis et al., 2005).

In order to further explain the regulation of 1, 2 – diazole and 2, 2, 2 – trifluoroethanol on lactate and ethanol production in the living *R. oryzae* culture, the modified Michaelis – Menten kinetics was determined using the productivity data reported in Table 4.5. The higher lactate formation rate revealed that under the operating conditions used in this study, glucose flux toward lactate production was preferable to that for ethanol production. The higher predicted $\nu_{max,\ app}$ values for lactate which were consistent to those shown in Table 4.5 confirmed that lactate production was promoted under sufficient oxygen supply and limited nitrogen source during the production phase. According to regression coefficient of the Lineweaver – Burk plots (data not shown) used to determine the modified Michaelis – Menten kinetics parameters, it could be also claimed that Michaelis – Menten model could be applied for determining the extrinsic kinetics of glucose uptake for the end products lactate and ethanol in the living *R. oryzae* containing several pools of enzymes (Skory et al., 2010). From the $\nu_{max,\ app}$ and $K_{m,\ app}$ values reported in Table 4.6, it can be claimed that 1, 2 – diazole and 2, 2, 2 – trifluoroethanol acted as the mixed competitive inhibitors of ADH.

Considering the affinity of glucose for each product (lactate and ethanol), it could be argued that *R. oryzae* most likely fermented glucose for lactate at high glucose concentration while it preferred producing ethanol at low glucose concentration in the control culture without the ADH inhibitors (Table 4.6). When 1, 2 – diazole was present in the culture, living *R. oryzae* behaved similarly to what occurred in the control culture but the decreasing $K_{m,\ app}$ for lactate while the increasing $K_{m,\ app}$ for ethanol were acquired compared with those determined in the control culture. This implied that 1, 2 – diazole as the NAD^+/NADH analog competitively bound at the coenzyme binding site of ADH. Although NADH is also

required in the bioconversion of glucose toward pyruvate for lactic acid production (Figs. 4.6 and 4.11), the affinity of 1, 2 – diazole to ADH was stronger than that to LDH as indicated by the higher $K_{m,\ app}$ for ethanol formation and the lower $K_{m,\ app}$ for lactate formation when this inhibitor was present in the fermentation compared with those found in the control culture in order to pursue the bioconversion of glucose to the end product ethanol. In addition, the increasing $\nu_{max,\ app}$ for ethanol formation revealed the dynamic of the bioconversion process in the living *R. oryzae*. It was presumed that when 1, 2 – diazole was present in the culture broth, due to its similar structure to NADH, *R. oryzae* responded to the excess flux of NADH and its analog by enhancing ADH expression in order to maintain the metabolic balance in the cells (Ma et al., 2012). This resulted in the increase in ADH concentration and eventually the increasing ethanol formation rate almost twice higher than that found in the control culture. On the other hand, $\nu_{max,\ app}$ for lactate formation in the culture with 1, 2 – diazole rather remained unchanged from that obtained from the control culture. Presumably, this was because 1, 2 – diazole has the lower binding affinity to LDH than that to ADH resulting in the unchanged concentration of NADH and its analog for LDH. Therefore, the living *R. oryzae* grown in both the control fermentation and that with 1, 2 - diazole most likely reduced NADH product for the bioconversion of pyruvate to lactate at the similar rate.

Increasing ethanol formation rate but decreasing affinity of glucose for the production of ethanol when 2, 2, 2 – trifluoroethanol was present in the culture broth is remarkable (Table 4.6). The very high value of $K_{m,\ app}$ of glucose flux toward ethanol formation indicated that 2, 2, 2 – trifluoroethanol highly competed with ethanol at the catalytic site; thus, somehow strongly preventing the conversion of acetaldehyde to ethanol. The kinetic results strongly confirmed that this inhibitor competitively bound at the catalytic site of ADH. From Figs. 4.10(c) and 4.11(a), it was believed that 2, 2, 2 – trifluoroethanol competitively bound to the catalytic site of ADH promoted the rapid reversible conversion of acetaldehyde and ethanol as observed from the high redox reaction rate ($\nu_{max,\ app}$) in order to maintain the NAD^+/NADH balance in the living *R. oryzae* culture. Though 2, 2, 2 – trifluoroethanol bound at the catalytic site of ADH which caused the increasing $K_{m,\ app}$ for glucose flux toward ethanol, the $K_{m,\ app}$ for glucose flux toward lactate was also increased. The reduction in the affinity of glucose for both ethanol and lactate when 2, 2, 2 – trifluoroethanol could be explained by the binding action of this inhibitor at the catalytic site which mimicked the high flux of ADH end product. This induced the

reversible conversion of the end product (ethanol) back to acetaldehyde to approach the new equilibrium during the dynamic growth and metabolism. However, the actual amount of ethanol present in the culture broth was still limited for the reversed conversion to acetaldehyde to reach the new equilibrium. Therefore, the living *R. oryzae* converted more glucose toward the glycolysis pathway.

Table 4.6 Apparent kinetics coefficients of glucose uptake for lactate and ethanol production in living *R. oryzae* grown in the simple glucose production medium containing ADH inhibitors at the concentration of 1 mM. The values were obtained from the modified Lineweaver-Burk plot.

Parameters	Maximum product formation rate, $\nu_{max,app}$ (g/L·h)	Affinity of glucose for product, $K_{m,app}$ (g/L)
Glucose flux toward lactate formation (Targeted LDH)		
CT	0.81	48.99
PZ (1)	0.68	30.32
TFE (1)	4.43	513.84
Glucose flux toward ethanol formation (Targeted ADH)		
CT	0.16	2.00
PZ (1)	0.30	11.60
TFE (1)	10.70	2,456.47

Remarks: CT control; PZ (1) 1, 2 – diazole at 1 mM; TFE (1) 2, 2, 2 – trifluoroethanol at 1 mM.

This revealed the fact that in the living culture when alcohol fermentative route was manipulated, the dynamic change in the central metabolite pyruvate pool affected lactate formation route as well. Due to the rapid reversible rate of ethanol conversion, this could also lead to the increasing $\nu_{max,\ app}$ for lactate formation (Umulis et al., 2005 and Dolega, 2010).

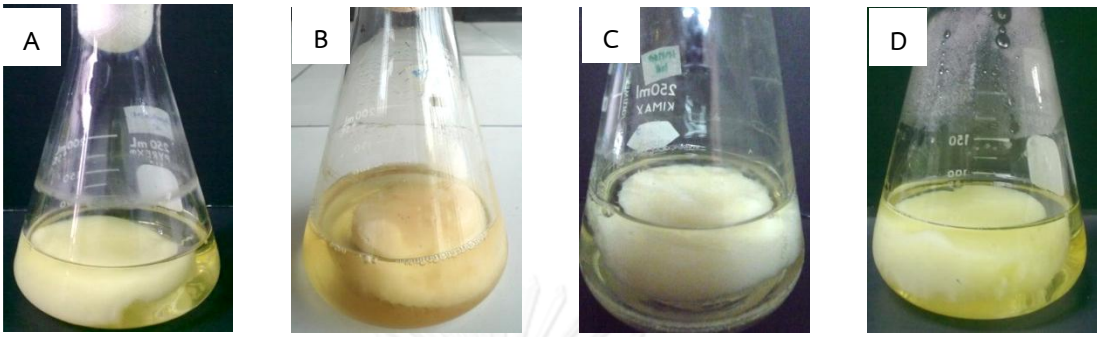
4.3 Manipulating pyruvate decarboxylase by addition of enzyme regulators during fermentation of *R. oryzae* to enhance lactic acid

4.3.1 Responses of *R. oryzae* on growth to the PDC regulators present in the fermentation medium

Culturing *R. oryzae* in the shake flask with the cotton cloth as the support for cell immobilization resulted in cell free fermentation broth as a result of spore germination and cell growth initiation only on the surface provided (Fig. 4.12). Compared to the submerged fermentation using free cells, immobilizing *R. oryzae* this way provided simple yet effective morphological control throughout the fermentation period. This resulted in higher cell density system with better mass transfer; thus, favored lactate production (Park et al., 1998; Tay and Yang, 2002; Chotisubhaananda et al., 2010).

Similar cell behavior appeared regardless to the presence of PDC regulators when immobilizing *R. oryzae* on the cotton cloth (Fig. 4.12). This implies that the selected PDC regulators added into the fermentation medium did not impair the cell growth and metabolism. In addition, immobilization rather protected the cell from the dramatic conditions from the environment.

Growth phase



Production phase

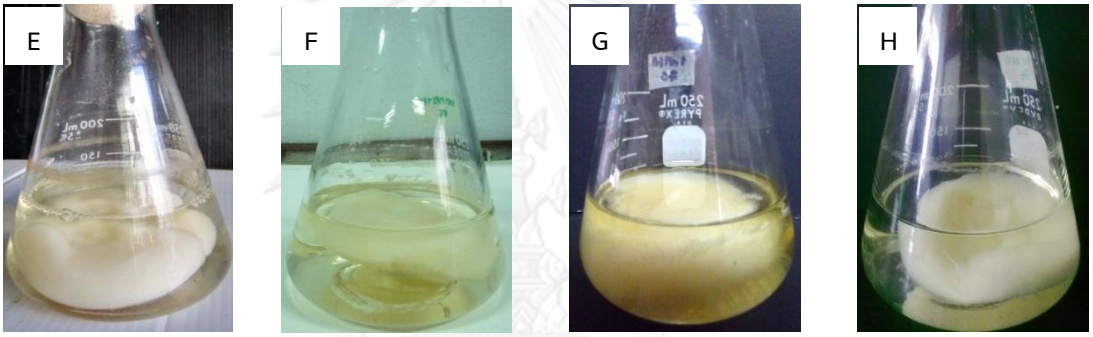


Figure 4.12 The unchanged morphology of immobilized *R. oryzae* on 5 × 5 cm^2 cotton cloth in the glucose based medium cultivated at 30 °C and 200 rpm was observed throughout the fermentation from the growth phase (A (control at 24 h growth), B (with 1 mM 4-methyl pyrazole), C (with 1 mM glyoxylic acid at 24 h growth), D (with 1 μM β-hydroxypyruvate at 24 h growth)) towards the production phase (E (control at 24 h production), F (with 1 mM 4-methylpyrazole at 24 h production), G (with 1 mM glyoxylic acid at 24 h production), H (with 1 μM β-hydroxypyruvate at 24 h production)).

4.3.2 End metabolite production from the living culture of *R. oryzae* with the presence of PDC regulators

Table 4.7 shows the fermentation kinetics of *R. oryzae* grown in the glucose medium. PDC regulator was added into the fermentation medium during both growth and production phases. Among 3 regulators studied, compared to the control fermentation, none of them showed significant effect on cell yield and productivity during the growth phase; thus, this can be initially claimed that these regulators did not alter the growth of the living *R. oryzae* and thus could be used in

fermentation. Lactate yield during the growth phase was found rather unchanged in the fermentation containing PDC regulators in regardless to their concentration whereas the productivity tended to change with the regulator concentration. Increasing glyoxylic acid concentration led to the increasing lactate productivity while lower lactate productivity was found when the concentration of 4-methylpyrazole was increased. In case of β-hydroxypyruvate, both lactate yield and productivity were increased compared with those found in the control fermentation in glucose medium. Ethanol yield were increased with the presence of glyoxylic acid in regardless of its concentration whereas the productivity was inclined with the increasing concentration. The presence of 4-methylpyrazole in the growth medium at 0.1 mM resulted in the lower ethanol yield and productivity compared to those found in the control fermentation but when the concentration was increased to 1.0 mM, it caused both yield and productivity dramatically increasing. β-hydroxypyruvate seemed to hinder ethanol production since the growth phase as observed from the lower yield and productivity compared to those obtained from the control fermentation.

Considering the fermentation kinetics during the production phase where lactate production was to be promoted with the limited cell growth and ethanol formation. It was found that slightly change in cell yield and productivity were observed during this phase in the fermentation containing the regulators when compared with those in the control fermentation. In case of lactate production, the increasing lactate yield was clearly observed when glyoxylic acid was present. The increasing concentration did not affect the yield but the productivity was enhanced at the higher concentration. The increasing concentration of 4-methylpyrazole from 0.1 mM to 1.0 mM caused the negative effect on lactate production as observed from lowered yield and productivity. Nonetheless, at the concentration of 0.1 mM, 4-methylpyrazole gave the highest lactate yield of 0.42 g/g glucose which is approximately 24% increasing yield compared with that obtained from the control fermentation. β-hydroxypyruvate did not show the influence on lactate yield and productivity as observed from the similar value to that from the control fermentation. Ethanol production was increased when glyoxylic acid was present in the fermentation. While the presence of 4-methylpyrazole at 0.1 mM hindered ethanol production as observed from the lower yield and productivity. On the other hand, further increasing the concentration of 4-methylpyrazole to 1.0 mM resulted in the increasing ethanol yield and productivity. The presence of β-hydroxypyruvate appeared to give the most effective way in controlling ethanol production in terms

of lower yield and productivity which are 23% and 29% decreasing yield and productivity compared with those obtained from the control fermentation.

The effect of PDC regulators on the metabolism of *R. oryzae* was also observed during the production phase of pregrown immobilized cells in the simple glucose medium in order to ensure no altered growth and metabolism by the regulators from the growth phase. From Table 4.8, cell yield was decreased when the regulator was added into the production medium. Among 3 regulators studied, β-hydroxypyruvate with the lowest concentration added showed the strongest effect on growth inhibition during the production phase (80% decreasing cell yield compared with that in the control fermentation). By this way, it was believed that the central metabolite, pyruvate, flux would be diverted to other metabolic routes. While increasing the concentration of glyoxylic acid and 4-methylpyrazole from 0.1 mM to 1.0 mM in the production medium did not lead to any change in cell yield.

Lactate yield was increased when the regulators were present during the production phase perhaps due to the diversion of pyruvate flux towards lactate production pathway as a result of growth inhibition. The strongest influence was exhibited when β-hydroxypyruvate was present in the production medium as observed from the increasing yield (0.45 g/g) of approximately 32% compared with the value obtained from the control fermentation (0.34 g/g). Increasing the concentration of glyoxylic acid and 4-methylpyrazole from 0.1 mM to 1.0 mM in the production medium did not further increase lactate yield.

Ethanol yield was increased when glyoxylic acid was added during the production phase. Increasing the concentration of glyoxylic acid resulted in the increasing ethanol yield. Similar phenomena in ethanol production was observed in the fermentation with 4-methylpyrazole at 1.0 mM whereas no significant increase in ethanol yield was found at the concentration of 0.1 mM.

Table 4.7 Yields and productivities of end products from fermentations by immobilized *R. oryzae* in the glucose medium containing PDC regulators cultivated at 30 °C and 200 rpm.

Regulators	Product yield (g/g glucose)						Productivity (g/L·h)					
	Growth phase			Production phase			Growth phase			Production phase		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.06±0.03	0.09±0.01	0.37±0.03	0.03±0.02	0.34±0.01	0.13±0.01	0.07±0.01	0.11±0.00	0.45±0.06	0.02±0.01	0.40±0.00	0.14±0.12
GA(0.1)	0.09±0.04	0.08±0.01	0.49±0.09	0.07±0.03	0.42±0.01	0.31±0.14	0.10±0.03	0.09±0.01	0.50±0.07	0.07±0.04	0.39±0.01	0.26±0.09
GA(1.0)	0.07±0.02	0.11±0.02	0.48±0.13	0.03±0.02	0.43±0.12	0.23±0.11	0.08±0.01	0.14±0.01	0.63±0.21	0.03±0.01	0.46±0.15	0.26±0.12
MPZ(0.1)	0.05±0.01	0.13±0.03	0.31±0.06	0.01±0.02	0.42±0.02	0.11±0.04	0.06±0.02	0.15±0.04	0.35±0.07	0.01±0.02	0.41±0.08	0.10±0.04
MPZ(1.0)	0.07±0.02	0.10±0.07	0.44±0.06	0.05±0.03	0.33±0.09	0.19±0.01	0.09±0.01	0.11±0.03	0.53±0.15	0.06±0.04	0.36±0.10	0.20±0.01
3-HP(1.0)	0.07±0.01	0.11±0.01	0.28±0.03	0.03±0.00	0.35±0.05	0.10±0.02	0.08±0.01	0.14±0.03	0.32±0.06	0.03±0.01	0.36±0.01	0.10±0.04

Remarks: CT = control; GA = glyoxylic acid; MPZ = 4-methylpyrazole; 3-HP = β-hydroxypyruvate; the numbers in the blanket represent the concentrations in mM for glyoxylic acid and MPZ and in μM for 3-HP.

In contrary to yields, there was no dramatic change in the productivity for cell and lactate in the fermentation with the regulators compared to the values obtained from control fermentation. But the increasing ethanol productivity was observed when the concentration of glyoxylic acid and 4-methylpyrazole were increased from 0.1 mM to 1.0 mM. The results shown in Table 4.8 revealed the stronger influence on both yield and productivity of glyoxylic acid compared with 4-methylpyrazole at the same concentration studied. β-hydroxypyruvate showed the least impact on ethanol production when added into the production phase.

Similarly to our previous work (Thitiprasert et al., 2011), the highest lactate yield of approximately 0.45 – 0.47 g/g glucose was obtained from the fermentation of pregrown *R. oryzae* containing 2,2,2-trifluoroethanol at 0.01 mM and β-hydroxypyruvate at 1.0 μM. This finding revealed the direct binding at the catalytic sites of the enzyme manipulated pyruvate flux towards lactate production.

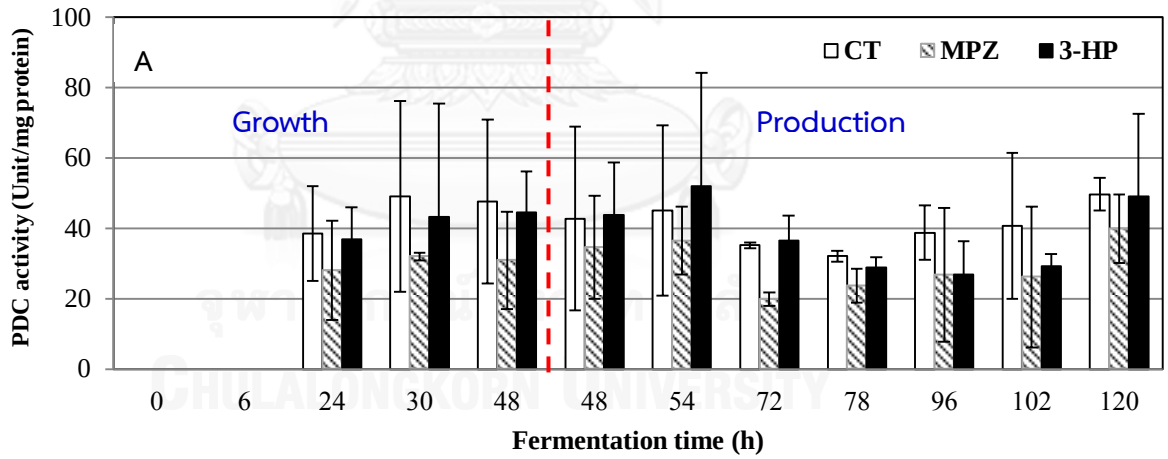
Table 4.8 Yields and productivities of end products from fermentations with the presence of PDC regulators at different concentrations by immobilized *R. oryzae* during the production phase controlled at 30 °C, 200 rpm, and pH 6.0. Immobilized *R. oryzae* was pregrown in the simple glucose medium for 48 h at 30 °C and 200 rpm.

Regulators	Product yield (g/g glucose)			Productivity (g/L·h)		
	Cell	Lactate	Ethanol	Cell	Lactate	Ethanol
CT	0.05±0.01	0.34±0.01	0.12±0.01	0.02±0.01	0.40±0.00	0.14±0.12
GA(0.1)	0.02±0.02	0.41±0.10	0.21±0.06	0.02±0.01	0.37±0.04	0.18±0.00
GA(1.0)	0.02±0.00	0.37±0.05	0.26±0.12	0.03±0.01	0.39±0.01	0.28±0.16
MPZ(0.1)	0.02±0.00	0.38±0.02	0.13±0.01	0.03±0.01	0.41±0.00	0.14±0.02
MPZ(1.0)	0.03±0.01	0.39±0.14	0.23±0.04	0.03±0.00	0.37±0.10	0.20±0.02
3-HP(1.0)	0.01±0.00	0.45±0.00	0.17±0.02	0.01±0.01	0.38±0.08	0.15±0.02

Remarks: CT = control; GA = glyoxylic acid; MPZ = 4-methylpyrazole; 3-HP = β-hydroxypyruvate; the numbers in the blanket represent the concentrations in mM for glyoxylic acid and MPZ and in μM for 3-HP

4.3.3 4-methylpyrazole and β-hydroxypyruvate and their role manipulating PDC, LDH, and ADH

From the fermentation kinetics shown in Tables 4.7-4.8, it is clear that 4-methylpyrazole at 0.1 mM and β-hydroxypyruvate at 1.0 μM showed the strong regulatory effects on pyruvate flux towards the end product routes; thus, they were selected for detailed study on the change in the enzyme activities when these 2 regulators were present during the fermentation. When the regulator was added into the fermentation since the growth phase, it was found that in regardless of the presence of the regulators, the activities of PDC and ADH(b) were increased with time while the activities of LDH and ADH(f) were increased until 30 h and later on they were dropped (Fig. 4.13). Except ADH(b), the presence of the regulators resulted in the lower activities of PDC, LDH, and ADH(f) compared with those in the control fermentation without the regulators. During the production phase, initially the enzyme activities were increased then started to drop when glucose was depleted. When the regulators were present, the lower activities of PDC, LDH, and ADH(f) were observed while ADH(b) activity on the other hand was increased as the response of *R. oryzae* to balance the flux at pyruvate pool.



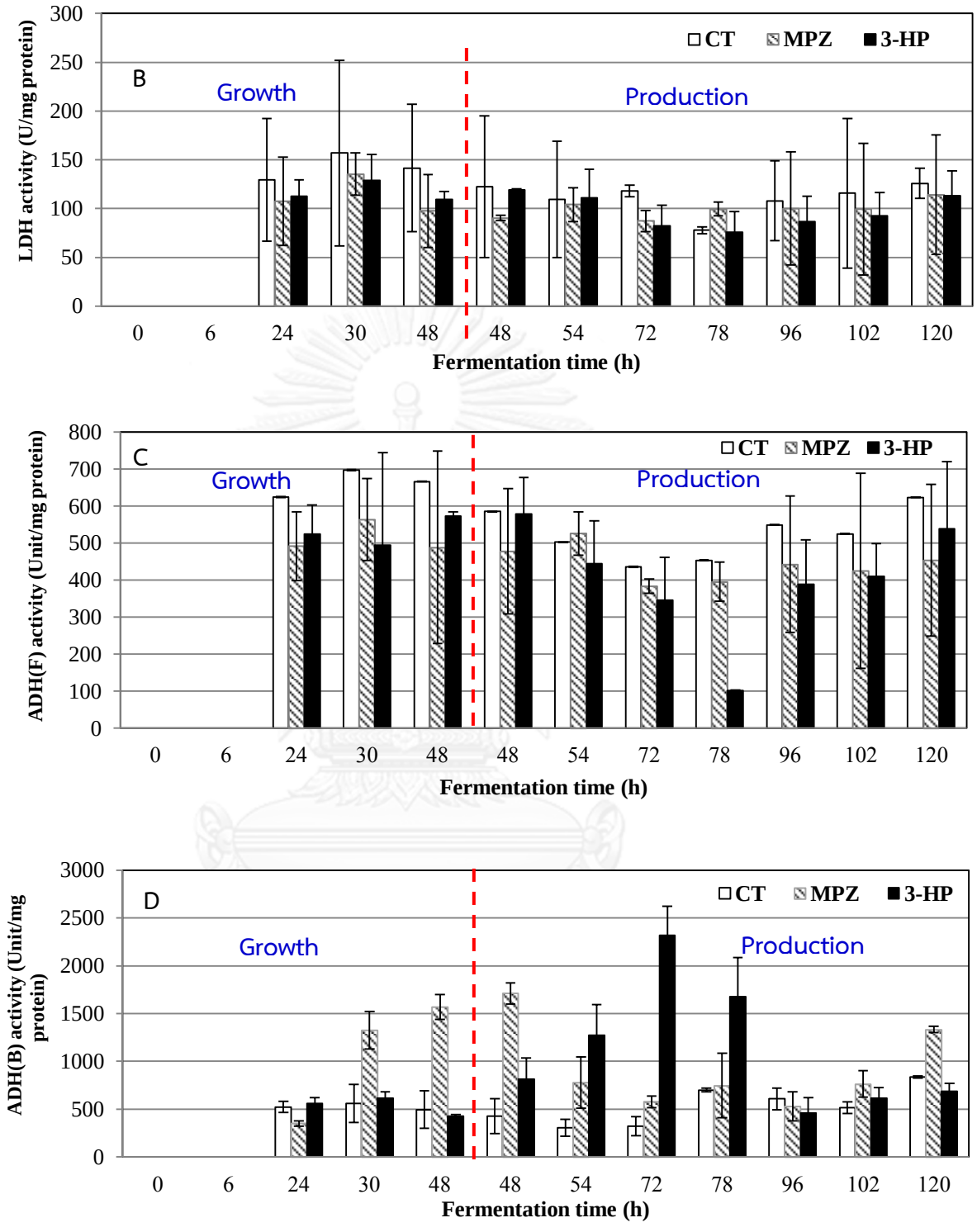
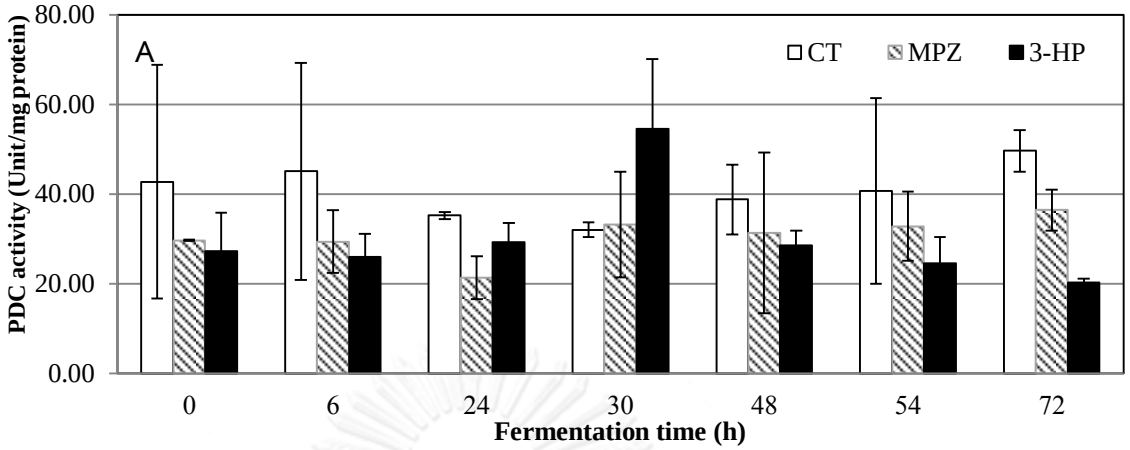
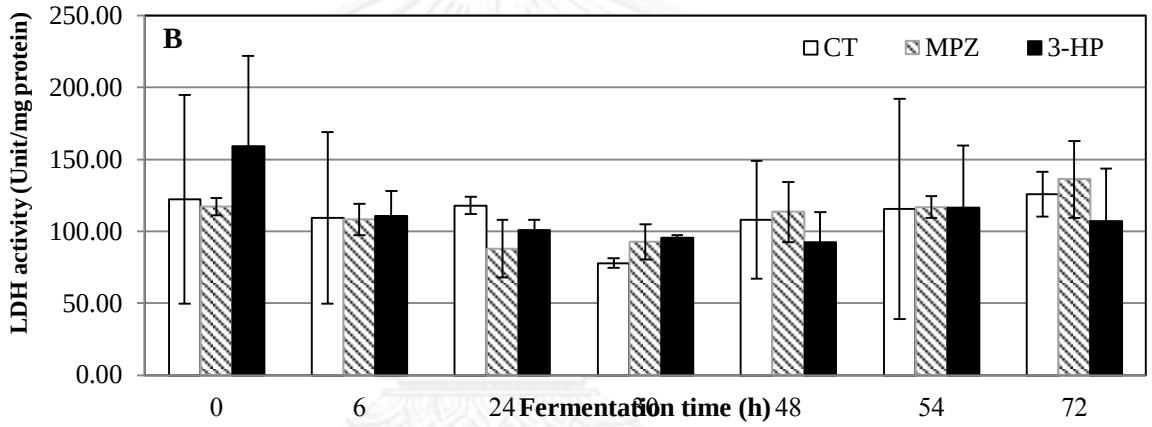


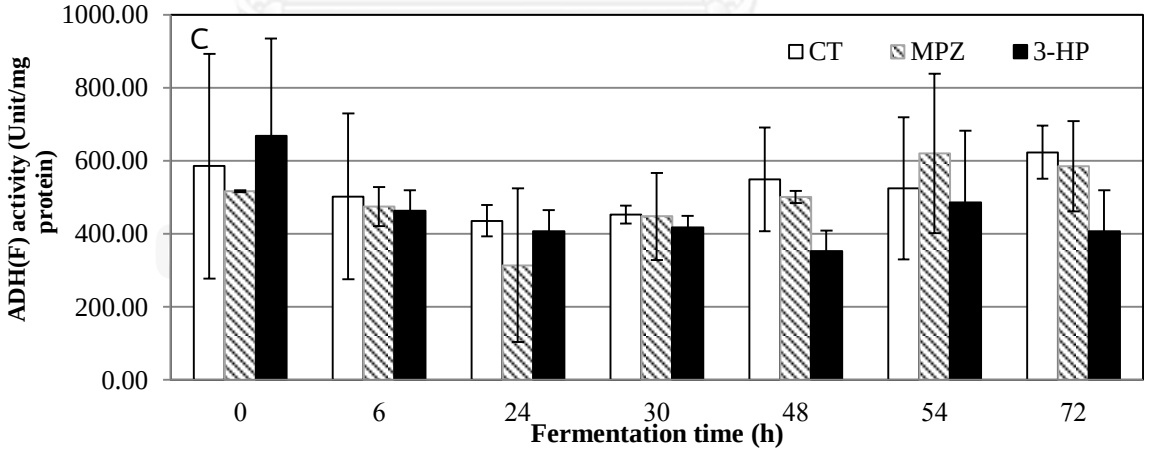
Figure 4.13 Specific activities of PDC (A), LDH (B), ADH(f) (C), and ADH(b) (D) during the fermentations by *R. oryzae* in the simple glucose medium (CT) containing 0.1 mM 4-methylpyrazole (MPZ) or 1.0 μM β-hydroxypyruvate (3-HP). Fermentation consisted of growth phase (48 h) where the culture was controlled at 30 °C and 200 rpm and

production phase where the culture was controlled at the same conditions as those in the growth phase with the additional pH control at 6.0 using 10 M NaOH.

In addition, *R. oryzae* was initially grown in the simple growth medium without the regulators. At the end of the growth phase, the growth medium was replaced by the production medium containing the regulators in order to observe the effects of the regulators presenting during the production phase only. From Fig. 4.14, the increasing enzyme activities were observed until glucose depletion. The similar evidence as observed earlier in the fermentation with the presence of the regulators since the growth phase was obtained. The activities of PDC, LDH, and ADH(f) from the fermentations with 4-methylpyrazole and 3-hydroxypyruvate were lower than those from the control fermentation whereas ADH(b) activity was higher when the regulators were present. Comparing the enzyme activities reported in Fig. 4.13-4.14, no significant difference in the enzyme activities was observed. This evidence implies that it is not necessary to add the regulators into the fermentation since the growth phase. Adding the regulators later in the production phase sufficiently controlled the pyruvate flux towards the end products.


A
CT
MPZ
3-HP
PDC activity (Unit/mg protein)
80.00
60.00
40.00
20.00
0.00
0
6
24
30
48
54
72
Fermentation time (h)


B
CT
MPZ
3-HP
LDH activity (Unit/mg protein)
250.00
200.00
150.00
100.00
50.00
0.00
0
6
24
Fermentation time (h)
48
54
72


C
CT
MPZ
3-HP
ADH(F) activity (Unit/mg protein)
1000.00
800.00
600.00
400.00
200.00
0.00
0
6
24
30
48
54
72
Fermentation time (h)

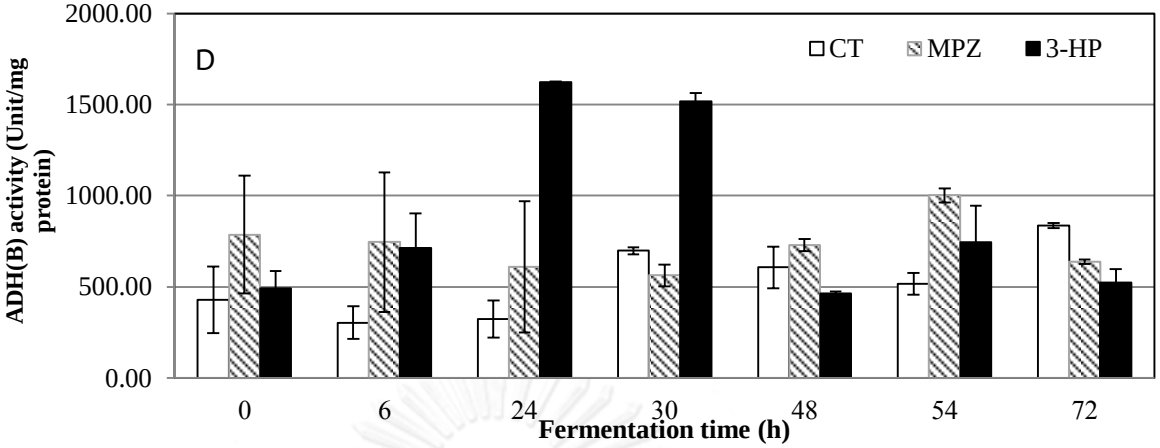


Figure 4.14 Specific activities of PDC (A), LDH (B), ADH(f) (C), and ADH(b) (D) during the production phase. Initially, *R. oryzae* was pregrown in the simple glucose medium for 48 h at 30 °C and 200 rpm. Later, the growth medium was discarded and replaced by the glucose based production medium (CT) containing 0.1 mM 4-methylpyrazole (MPZ) or 1.0 μM β-hydroxypyruvate (3-HP). Fermentation was prolonged for another 72 h at the same operating conditions with the additional pH control at 6.0 using 10 M NaOH.

4.3.4 4-methylpyrazole and β-hydroxypyruvate and their role as the competitive PDC inhibitors

In the metabolic pathway of *R. oryzae*, ethanol is produced as the results of the conversion of pyruvate by PDC and ADH. PDC is the first key enzyme that catalyzes the decarboxylation of pyruvate into acetaldehyde and CO_2. Acetaldehyde is subsequently reduced to ethanol by ADH. Considering PDC, it has been reported in various organisms, including plants, yeasts, some bacteria, and fungi (Kutter et al., 2007; Açar et al., 2007; Skory, 2003). The previous study on PDC gene induction in *R. oryzae* showed the highly similar sequences of the subunits of *R. oryzae* PDC to those in yeast (approx. 40%) and while the sequences were partially matched with that found in bacterial PDC (approx. 29%) (Açar et al., 2007; Skory, 2003). *R. oryzae* PDC is found in the cytosolic site of the fungi. PDC is the isoenzyme that can be found in 2 different native forms, those are dimers and tetramers. PDC reaction requires thiamine diphosphate (ThDP) and 2 Mg^{2+} as the cofactors (Kutter et al., 2006). Between the pH of 6.5-8.3, the amounts of dimer and tetramer are in equilibrium. The tetrameric form is activated at pH 6.5 (Açar et al., 2007 and Skory, 2003). When increasing the pH to alkali pH, 2 cofactors are dissociated rapidly from the enzyme resulting in the conformation change to the predominant dimer which is

more stable (Açar et al., 2007; Kutter et al., 2007). The dimer is created from 2 subunits of PDC tightly assembled. Within the dimer subunit, cofactors Mg^{2+} and ThDP are bound to the interface (Fig. 4.15). When pyruvate is accumulated in the cytosol, 2 units of PDC dimers are formed to a loose tetramer with the hydrogen bond and salt bridge (Furey et al., 1998).

The reaction sequence of the conversion of pyruvate by PDC is described from the binding of the substrate with the coenzyme at the catalytic site (Fig. 4.16B). The structure of 4-methylpyrazole, as clearly seen in Fig. 4.16A, resembles to the thiazolium ring in ThDP; thus, competitively attracting the pyruvate pool to be entering the ethanol production route. This subsequently resulted in incomplete PDC enzyme substrate binding, low acetaldehyde flux for the conversion by ADH to the end product ethanol. From Fig. 4.13-4.14, the lower PDC and ADH(f) but the higher ADH(b) activities confirm the action of 4-methylpyrazole by limiting the conversion of pyruvate to acetaldehyde. *R. oryzae* recognized the insufficient acetaldehyde pool; therefore, the backward direction of ADH dominated in order to balance the flux. The decreasing LDH activity can be explained by the similar cyclic structure of 4-methylpyrazole to that in NADH, the cofactor of LDH. Nonetheless, no report on this evidence can be found in the literatures. Further study is required to summarize this finding. Unlike 4-methylpyrazole, β-hydroxypyruvate directly competed with pyruvate to bind at the catalytic sites of PDC and LDH; thus resulting in the lower enzyme activities and lactate production slightly (Fig. 4.13-4.14 and Table 4.8-4.9). Similarly to what observed in the fermentation with the presence of 4-methylpyrazole, the lower ADH(f) but the higher ADH(b) activities were found. This confirmed the evidence of low acetaldehyde pool in the living culture; therefore, *R. oryzae* attempted to reverse the direction of ADH reaction to acetaldehyde instead of the end product ethanol. This eventually led to the lower ethanol production.

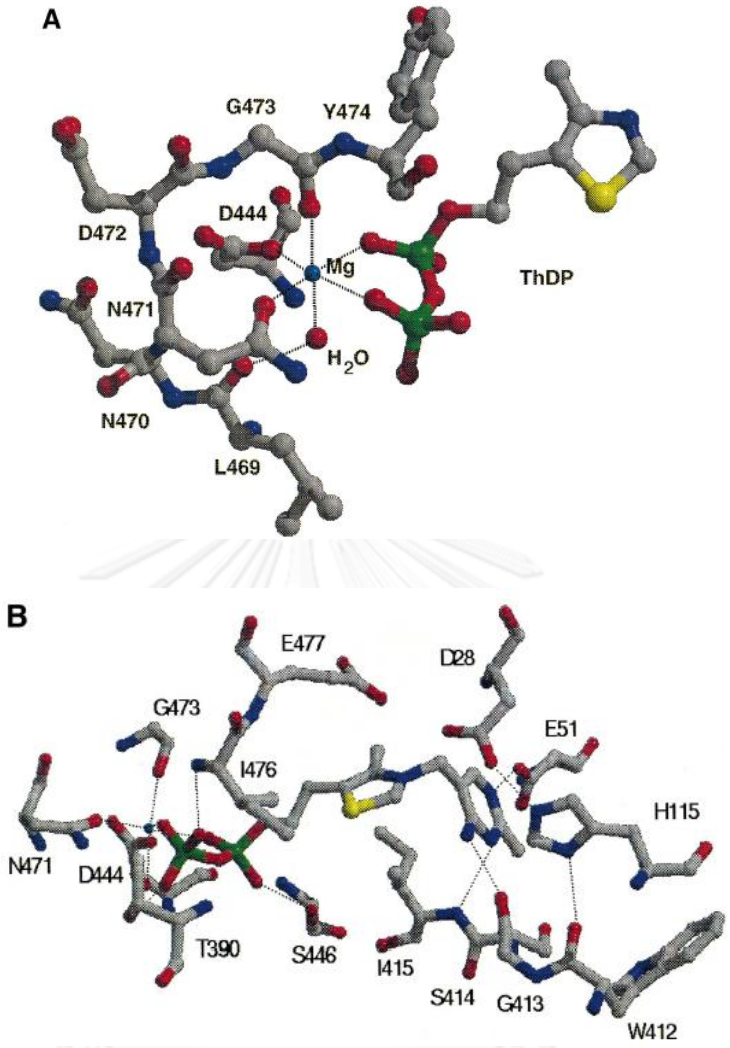


Figure 4.15 Structure of PDC binding sites to ThDP and Mg^{2+} (A) and the structural environment of PDC showing the interaction between ThDP and the protein, and neighboring residues likely to be involved in catalysis or stabilization of the ThDP conformation (B) (Furey et al., 1998).

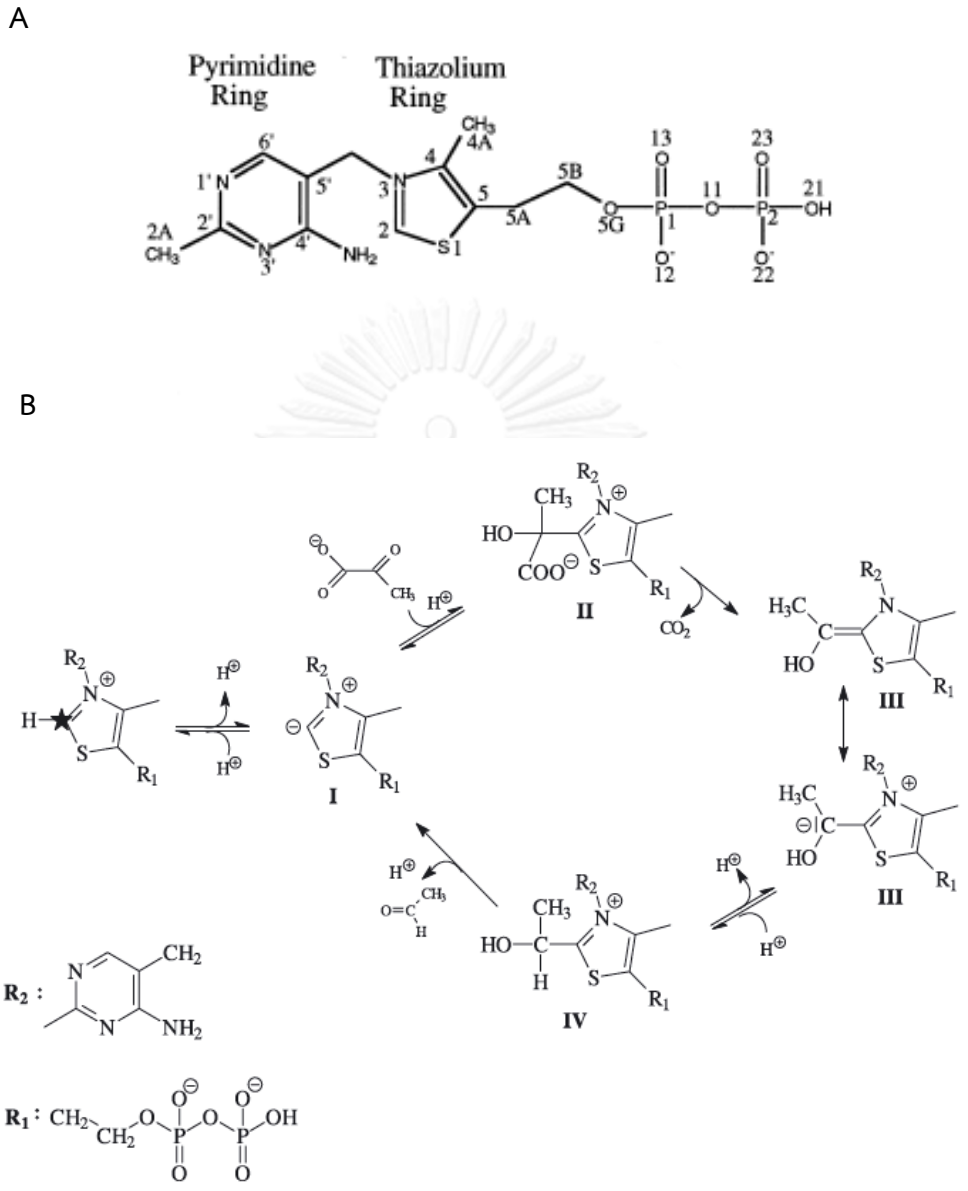
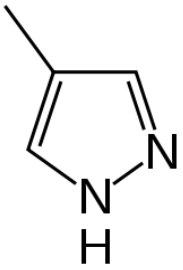


Figure 4.16 Thiamine diphosphate (vitamin B1) structure (A) and its function as the substrate binding site (B) during the catalytic cycle of PDC. Pyruvate conversion is initiated from the substrate binding at the cofactor (ThDP) by deprotonation of the C2 atom of the thiazolium ring (marked by an asterisk). The resulting ylid of ThDP (I) then attacks the carbon atom of the carbonyl group of the substrate pyruvate, generating lactyl ThDP (II). The subsequent decarboxylation of II results in the α-carbanion-enamine of ThDP (III). III is then protonated and the hydroxyl ethyl ThDP (IV) is formed and acetaldehyde is readily released to complete the cycle (Furey et al., 1998 and Kutter et al., 2006).

A



B

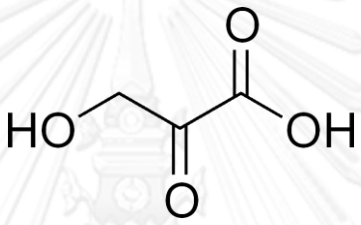


Figure 4.17 2 PDC regulators present during the fermentation by *R. oryzae* in this study. 4-methylpyrazole (A) resembles to the thiazolium ring in ThDP, the PDC cofactor. β-hydroxypyruvate (B) with the similar structure to pyruvate acts as the competitive inhibitor binding to the catalytic site at the thiazolium ring in ThDP.

4.4 The role of pyruvate decarboxylase and alcohol dehydrogenase regulators on the fermentation behavior of immobilized *R. oryzae* NRRL395 for enhancing L(+)-lactic acid production in a static bed bioreactor

The potential PDC and ADH regulators were obtained from the shake flask culture of immobilized *R. oryzae.* The results in shake flask showed that 4 regulators at the particular concentrations were able to regulate the targeted enzymes as well as increased L(+)-lactic acid production. Those included 1mM 1,2-diazole, 1mM 2,2,2-trifluoroethanol, 1 μM β-hydroxypyruvate, and 0.1mM 4-methylpyrazole. ADH and PDC regulators were added into the fermentation medium either at the beginning of the growth phase or later on during the production phase. The results shown earlier indicated that adding the regulators during the fermentation sufficiently controlled the flux of pyruvate towards lactate production as observed from the subsequently increasing lactate yield and productivity. The specific enzyme activities as well as the affinity to substrates determined using the modified Michaelis Menten equation revealed that the further improved lactate production by diverting pyruvate flux

from entering the ethanol fermentative pathway could be achieved under the optimized dissolved oxygen level. Improved L(+)-lactic acid production in a static bed bioreactor was investigated. The concentration effects of the selected regulators on growth and end metabolite production as well as the key enzyme expression were observed.

4.4.1 Fungal growth and morphology in the medium containing PDC and ADH regulators

Figure 4.18 shows fungal growth and morphology in the glucose medium containing enzyme regulator. It is clear that immobilizing *R. oryzae* spores and allowing them to germinate on the sheet of cotton cloth affixed at the static matrix provided the easy way to control the morphology that allowed easy bioreactor operation. By this way, *R. oryzae* mycelia were completely immobilized on the cotton matrix provided no cell was found in the fermentation medium or any bioreactor surfaces. In addition, this technique could improve oxygen transfer which subsequently reduced byproduct ethanol formation and also increased L(+)-lactic acid production. Similar results were observed by Chotisubha-anandha and coworker as well as Tay and Yang (Tay and Yang, 2002 and Chotisubha-anandha et al., 2011).

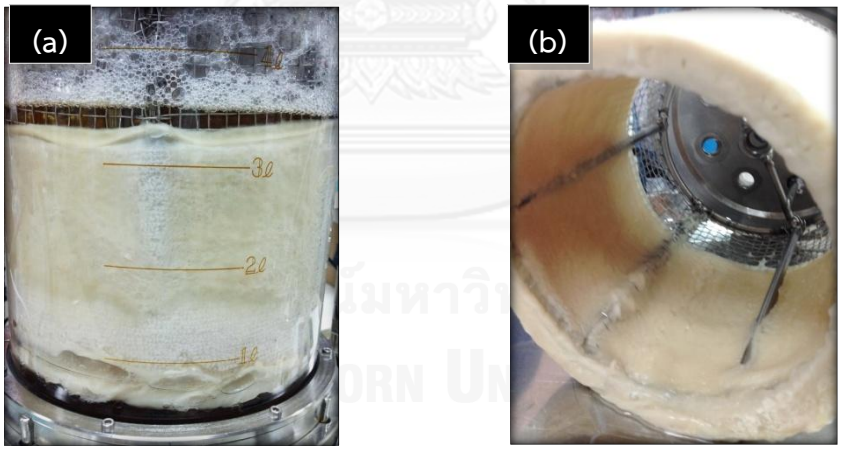
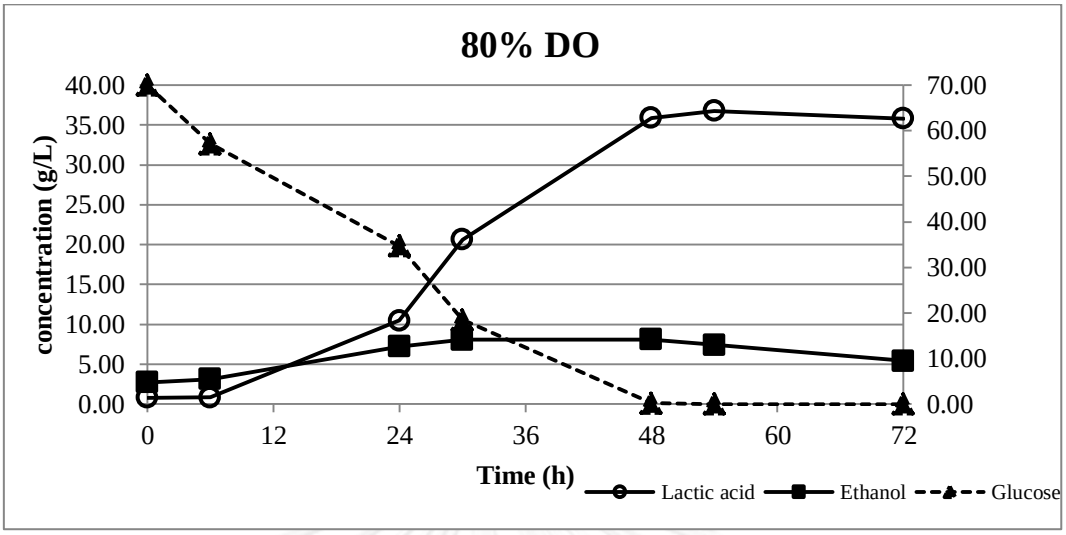


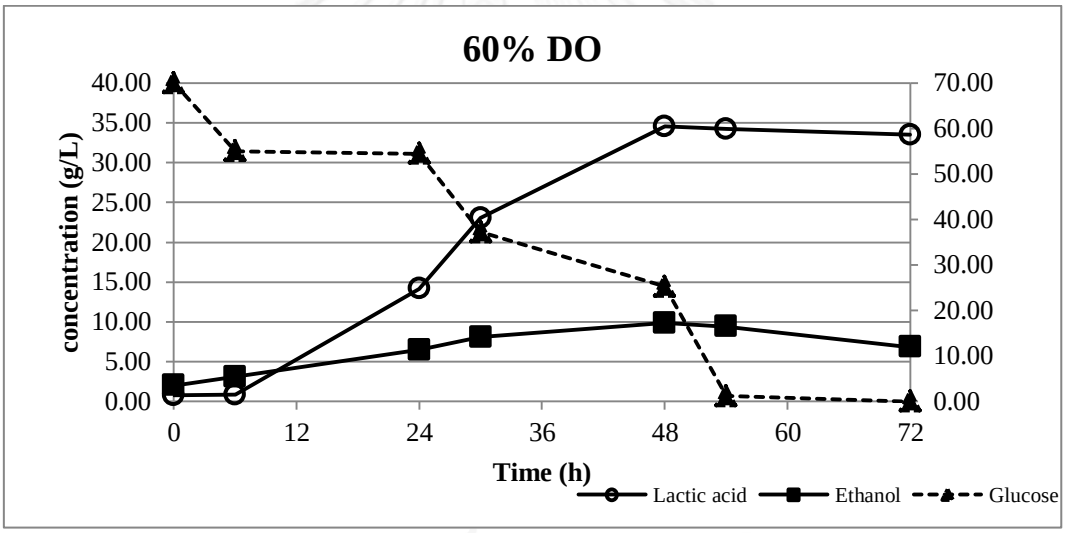
Figure 4.18 Appearance of immobilized *R. oryzae* on a sheet of cotton cloth affixed at the static bed in the stirred fermentor (a) and its thin layer of immobilized mycelia attached on the cotton cloth surface at the end of the fermentation (b).

4.4.2 Effect of different dissolve oxygen concentrations and enzyme regulators on fermentation kinetics of immobilized *R. oryzae*

The DO concentrations were varied within the range of 40 to 80 % by varying the agitation rate from 100 to 300 rpm and adjusting the oxygen enriched air

rate to obtain the particular. The results indicated that DO concentration played an important role on L(+)-lactic acid production in *R. oryzae*. As shown in figure 4.19, the higher lactic acid and less ethanol production were obtained at the increasing DO level during the cultivation of immobilized *R. oryzae* grown in the simple glucose medium (control) as well as the medium containing enzyme regulators. The findings were consistent with those reported in the previous studies and the hypothesis indicating that lactic acid production by *R. oryzae* is an aerobic process and DO plays the significant role on product formation (Tay and Yang, 2002). When reducing the DO level from 80 % to 40 %, ethanol production was significantly increased in all treatments. This was consistent with several studies reporting the promotion of ethanol formation in oxygen limiting environment as well as the reduction of lactic acid production in *R. oryzae* (Tay and Yang, 2002; Skory, 2003; Fu et al., 2009; Chotisubha-anadha et al., 2011; and Zhang et al., 2012). Similar cell biomass production was observed in control fermentation as well as fermentation containing each regulator under different DO concentrations. At the same DO level, compared to the control fermentation, in the fermentation containing the regulators, the higher lactic acid production was obtained as the result of the regulators competed with either their specific cofactor or substrate at the targeted enzyme binding sites; thus allocated pyruvate flux towards lactate production. Maximum lactic acid yield and productivity (0.63 g/g glucose and 0.98 g/L·h, respectively) with the concentration of 43.14 g/L were obtained from the fermentation containing 1 mM 2,2,2-trifluoroethanol as well as less ethanol production with the yield and productivity of 0.04 g/g glucose and 0.09 g/L·h, respectively (Table 4.9). This was about 24 % increasing yield compared with that of the control fermentation (0.51 g/g glucose). This would be perhaps due to its resembled structure to ethanol analog; thus, when 2,2,2-trifluoroethanol was present in the medium, the cell metabolism could respond to the excess ethanol, leading to pyruvate which was fluxed to lactic acid production instead.





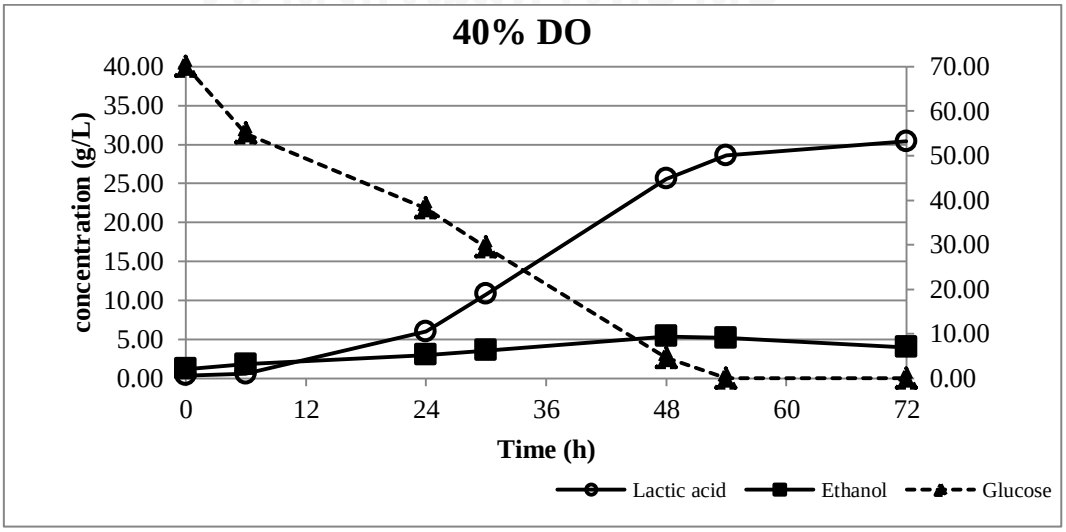


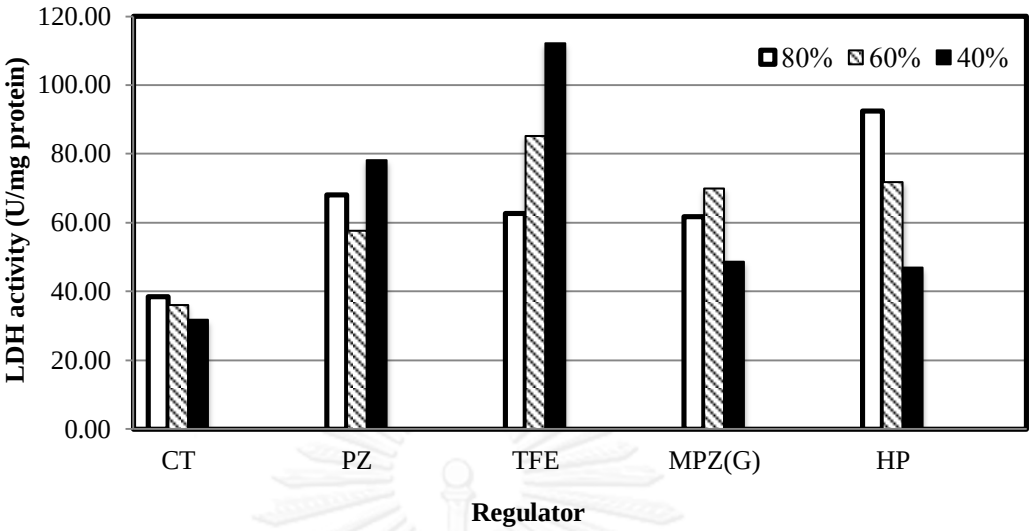
Figure 4.19 The fermentation profiles of lactic acid fermentation by immobilized *R. oryzae* grown in the simple glucose medium (control).

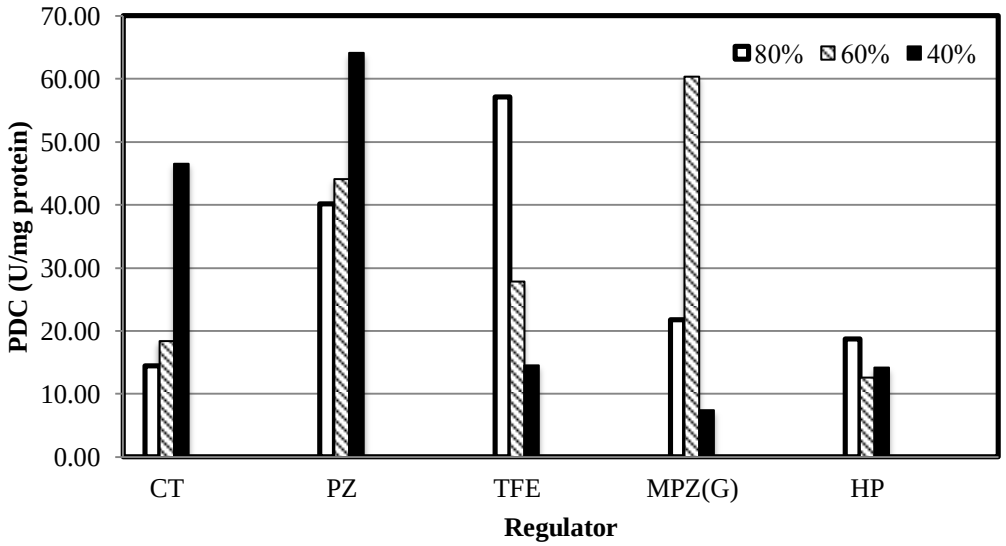
Table 4.9 Product yields and productivities during the fermentation medium containing enzyme regulators in the culture of *R. oryzae* in a static bed bioreactor under different DO concentrations

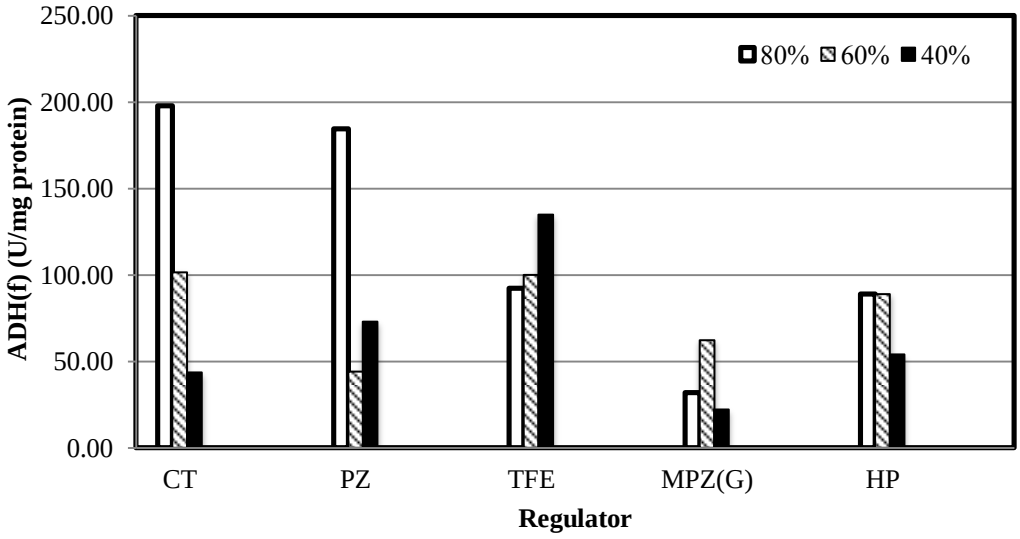
Regulators	CT			1mM PZ			1mM TFE			0.1 mM MPZ			1 μM HP		
DO concentrations	40	60	80	40	60	80	40	60	80	40	60	80	40	60	80
Yield (g/g glucose)															
Cell biomass	0.07	0.07	0.06	0.08	0.06	0.07	0.06	0.07	0.07	0.06	0.07	0.09	0.05	0.06	0.06
Lactic acid	0.41	0.48	0.51	0.44	0.50	0.58	0.54	0.55	0.63	0.50	0.54	0.54	0.42	0.51	0.53
Ethanol	0.11	0.12	0.06	0.11	0.09	0.05	0.08	0.08	0.04	0.10	0.07	0.05	0.10	0.07	0.05
Productivity (g/L·h)															
Cell biomass	0.08	0.07	0.06	0.08	0.07	0.07	0.06	0.08	0.08	0.06	0.08	0.09	0.05	0.07	0.06
Lactic acid	0.49	0.66	0.70	0.51	0.67	0.75	0.71	0.75	0.98	0.60	0.77	0.79	0.56	0.70	0.77
Ethanol	0.14	0.17	0.08	0.16	0.12	0.08	0.10	0.10	0.09	0.13	0.09	0.10	0.13	0.09	0.08

4.4.3 Effect of PDC and ADH regulators on enzyme target activities in living culture of *R. oryzae*

After the batch fermentation was carried out (approx. 72h), fresh mycelia were extracted for analyzing LDH, PDC, and ADHs activities. Figure 4.20 shows the changes of enzyme activities under different cultivation conditions. It was found that all of 4 regulators could increase the activity of LDH in various DO concentrations, comparing with control fermentation. This finding supported the previous results on increasing lactate yield in the culture of *R. oryzae* grown in the fermentation containing these regulators. Additionally, all of these 4 regulators can be divided into 2 groups, including substrate and cofactor analog. Among these 2 groups, substrate analog became more profound to manipulate the targeted enzyme activities than cofactor analogs. The members of substrate analog studied consists of 2,2,2-trifluoroethanol and β-hydroxypyruvate which have the resembled structures to ethanol and pyruvate, respectively. When DO value was increased from 40 to 80 %, β-hydroxypyruvate lowered PDC, ADH(f) and ADH(b) activities, on the other hand, leading to an increase in LDH activity. This would be indicated that at the high DO value, β-hydroxypyruvate could compete with pyruvate in order to bind with PDC; thus, it does directly influence the enzyme expression in the living culture of *R. oryzae*. While 2,2,2-trifluoroethanol exhibited the reverse effect on enzyme expression compared with β-hydroxypyruvate. Under increasing DO value from 40 to 80 %, LDH, ADH(f), and ADH(b) activities were decreased while PDC activity was contrary increased. This would be explained that at the lower DO level, 2,2,2-trifluoroethnaol played as an ADH regulator; hence, when increasing DO level, this regulator could not compete with ethanol for binding at the active site of ADH which resulting in pyruvate flux driven towards ethanol fermentative pathway. However, it was not only the reverse direction of ethanol to acetaldehyde formation was still higher, the lower ADH(f) activity was found. This implied that low accumulation of acetaldehyde formation was occurred, leading to the lower ethanol production by decreasing PDC activity (Fig. 4.20 and Table 4.9). The group of enzyme cofactor analog include 1,2-diazole and 4-methylpyrazole which are the NADH/NAD^+ analog. These 2 regulators were expected that they could compete to cofactor binding enzyme active site; particularly, increasing DO levels might suppress enzyme target (PDC, ADH(f), and ADH(b)) activities. However, it was found that both of such cofactor analogs did not directly influence the enzyme expression in living culture of *R. oryzae* (Fig. 4.20).


120.00
100.00
80.00
60.00
40.00
20.00
0.00
LDH activity (U/mg protein)
80%
60%
40%
CT
PZ
TFE
MPZ(G)
HP
Regulator


70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
PDC (U/mg protein)
80%
60%
40%
CT
PZ
TFE
MPZ(G)
HP
Regulator


250.00
200.00
150.00
100.00
50.00
0.00
ADH(f) (U/mg protein)
80%
60%
40%
CT
PZ
TFE
MPZ(G)
HP
Regulator

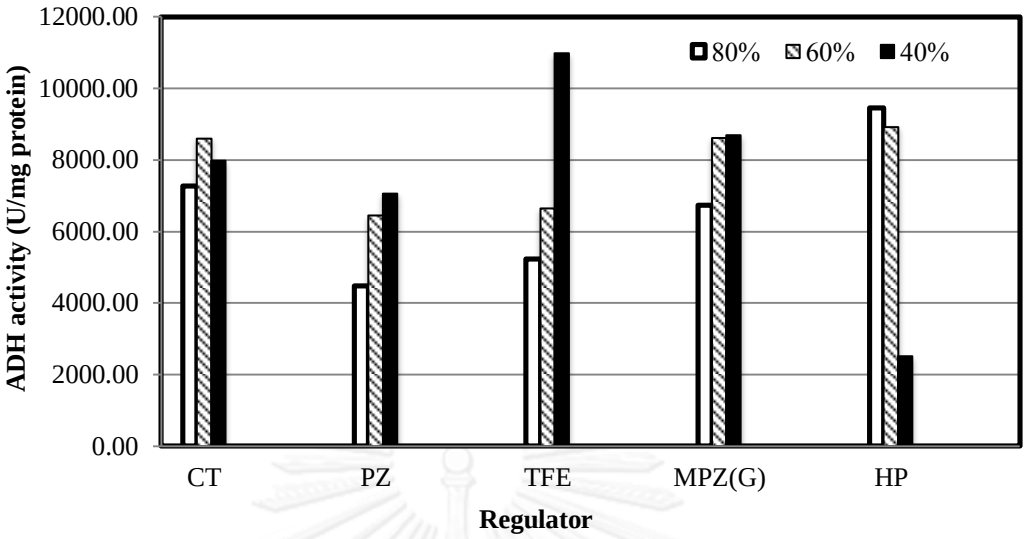


Figure 4.20 The specific activities of LDH, PDC, ADH(f), and ADH(b) during the culture of *R. oryzae* in the fermentation medium containing 1mM 1,2-diazole (PZ); 1mM 2,2,2-trifluoroethanol (TFE); 0.1 mM 4-methylpyrazole (MPZ); and 1μM β-hydroxypyruvate (HP) under different DO concentrations (40, 60, and 80%)

CHAPTER V

CONCLUSION AND SUGGESTION

5.1 Conclusion

Lactic acid fermentation by *R. oryzae* usually suffers from the formation of byproduct ethanol. There are two key enzymes responsible to ethanol production. These are PDC and ADH. It was hypothesized that decreasing ethanol could be achieved by limiting pyruvate flux towards ethanol fermentative route. This could be simply done by regulating PDC and ADH. The competitive enzyme inhibitors were selected from the in vitro study reported in the literatures. Those included 1,2-diazole, 2,2,2-trifluoroethanol, iodoacetic acid, N-ethylmaleimide, 4,4'-dithiodipyridine, and 4-hydroxymercury benzoic acid for regulating ADH. While 4-methylpyrazole, glyoxylic acid, and β-hydroxypyruvate were selected to regulate PDC. The regulation of the 2 key enzymes, PDC and ADH, was initially performed in the shake flask culture of the living immobilized *R. oryzae*. 1,2-diazole with the resemble structure to NADH and 2,2,2-trifluoroethanol, the competitive inhibitor binding at the catalytic site of ADH effectively regulated the pyruvate flux towards lactate production. Similarly, 4-methylpyrazole and β-hydroxypyruvate, the PDC regulators, gave the improved lactate production when added into the production medium.

Nonetheless, the addition of such mentioned enzyme regulators during the cultivation of living culture of *R. oryzae* instead of only limited ethanol fermentative pathway, they caused the dynamic changes in pyruvate flux to the end product routes as observed from the change in the specific enzyme activities. To further observe the effects of the enzyme regulators, fermentation was carried out in the static bed bioreactor at various dissolved oxygen concentrations. The results revealed that the influence of regulators was more profound at the higher dissolved oxygen concentration. The increasing lactate yield but the lower ethanol formation was obtained when the regulators were present during the production phase at the high DO level of 80%.

From the results obtained in this study, it can be concluded that the regulators added to suppress ethanol fermentative pathway not only affected one specific targeted enzyme, the other metabolic routes were also indirectly

manipulated as the cells attempted to balance the metabolic fluxes of substrate conversion toward the end products.

5.2 Suggestion for future work

To increase the productivity of lactic acid, increasing NADH/NAD^+ regeneration rate can drive the pyruvate flux toward the end product lactate. The fermentation with the external NADH/NAD^+ enhancer, for example, would help accelerate the fermentation rate.

REFERENCES

Abdel-Rahman, M. A., Tashiro, Y., and Sonomoto, K. (2013) Recent advances in lactic acid production by microbial fermentation processes, *Biotechnology Advances* 30, 877-902.

Acar, S., Yucel, M. and Hamamci, H. (2007) Purification and characterization of two isozymes of pyruvate decarboxylase from *Rhizopus oryzae*, *Enzyme and Microbial Technology* 40, 675-682.

Bai, D. M., Jia, M. Z., Zhao, X. M., Ban, R., Shen, F., Li, X. G., and Xu, S. M. (2003) L(+)-lactic acid production by pellet-form *Rhizopus oryzae* R1021 in a stirred tank fermentor, *Chemical Engineering Science* 58, 785-791.

Bailey, J.E., and Ollis, D.F. (1986) *Biochemical Engineering Fundamentals International ed.,* McGraw – Hill Book Company, Singapore.

Budavari, S., O'Neil, M. J., Smith, A. and Heckelman, P. E. (Ed.) (1989) *The merck index*,
11th ed., The United States Pharmaceutical Merck & Co, NJ.

Bulut, S., Elibol, M. and Ozer, D. (2004) Effect of different carbon sources on L(+)-lactic acid production by *Rhizopus oryzae*, *Biochemical Engineering Journal* 21, 33–37.

Bulut, S., Elibol, M., and Ozer, D. (2009) Optimization of process parameters and culture medium for L-(+)-lactic acid production by *Rhizopus oryzae*, *Journal of Chemical Engineering of Japan* 42, 589-595.

Büyükkileci, A. O., Hamamcı, H., and Yucel, M. (2006) Lactate and ethanol productions by *Rhizopus oryzae* ATCC 9363 and activities of related pyruvate branch point enzymes, *Journal of Bioscience and Bioengineering* 102, 464-466.

Chotisubha – anandha N., Thitiprasert S., Tolieng V., and Thongchul N. (2011) Improved oxygen transfer and increased L – lactic acid production by morphology control of Rhizopus oryzae in a static bed bioreactor, *Bioprocess and Biosystems Engineering* 34, 163 – 172.

Datta, R., Tsai, S. P., Bonsignor, P., Moon, S. and Frank, J. (1995) Technological and economic potential of poly(lactic acid) and lactic acid derivatives, *FEMS Microbiology Reviews* 16, 221–231.

Datta, R. and Henry, M. (2006) Lactic acid: recent advances in products, processes, and technologies-a review, *Journal of Chemical Technology and Biotechnology* 81, 1119–1129.

Ding, S. and Tan, T. (2006) L-lactic acid production by *Lactobacillus casei* fermentation using different fed-batch feeding strategies, *Process Biochemistry* 41, 1451–1454.

Dolega A. (2010) Alcohol dehydrogenase and its simple inorganic models, *Coordination Chemistry Reviews* 254, 916 – 937.

Dong, X. Y., Bai, S. and Sun, Y. (1996) Production of L(+)-lactic acid with *Rhizopus oryzae* immobilized in polyurethane foam cubes, *Biotechnology Letter* 18, 225–228.

Ellis, D. H. (1997) Zygomycetes, In *Topley and Willson's Microbiology and Microbial Infections*, pp. 247-277, Edward Arnold London, 1997.

Efremenko, E. N., Spiricheva, O. V., Veremeenko, D. V., Baibak, A. V., and Lozinsky, V.I. (2006) L(+)-lactic acid production using poly(vinyl alcohol)-cryogel-entrapped *Rhizopus oryzae* fungal cells, *Journal of Chemical Technology and Biotechnology* 81, 519-522.

Furey, W., Arjunan, P., Chen, L., Sax, M., Guo, F., and Jordan, F. (1998) Review: Structure-function relationships and flexible tetramer assembly in pyruvate decarboxylase revealed by analysis of crystal structures, *Biochimica et Biophysica Acta* 1385, 253-270.

Ganguly, R., Dwivedi, P., and Singh, R. P. (2007) Production of lactic acid with loofa sponge immobilized *Rhizopus oryzae* RBU2-10, *Bioresource Technology* 98, 1246-1251.

Garg, N. and Hang, Y.D. (1995) Microbial production of organic acids from carrot processing waste, *Journal of Food Science and Technology* 32, 119-121.

Gheinani, A. H., Jahromi, N. H., Feuk-Lagerstedt, E., and Taherzadeh, M. J. (2011) RNA silencing of lactate dehydrogenase gene in *Rhizopus oryzae*, *Journal of iRNA and Gene Silencing* 7, 443–448.

Ghosh, B., and Ray R. R., Current commercial perspective of *Rhizopus oryzae* : A Review, *Journal of Applied Sciences* 11, 2470-2486.

Goldstein, D. B., and Pal, N. (1971) Comparison of pyrazole and 4 – methylpyrazole as inhibitors of alcohol dehydrogenase: their potency, toxicity and duration of action in mice, *The Journal of Pharmacology and Experimental Therapeutics* 178, 199 – 203.

Gryganskyi, A. P., Lee, S. C., Litvintseva, A. P., Smith, M. E., Bonito, G., Porter, T. M., Anishchenko I. M., Heitman, J., and Vilgalys, R. (2010) Structure, Function, and Phylogeny of the mating locus in the *Rhizopus oryzae* complex, *PLOS ONE* 5, 1-12.

Hang, Y. D., and Suntornsuk, W. (1994) Strain improvement of *Rhizopus oryzae* for production of L(+)-lactic acid and glucoamylase, *Letters in Applied Microbiology* 19, 249-252.

John, R. P., Nampoothiri, M. K., and Pandey, A. (2007) Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives, *Applied Microbiology and Biotechnology* 74, 524-534.

Kosakai, Y., Park, Y.S. and Okabe, M. (1997) Enhancement of L(+)-lactic acid production using mycelial flocs of *Rhizopus oryzae*, *Biotechnology and Bioengineering* 55, 461–470.

Krull, R.,Wucherpfennig, T., Esfandabadia, M. E., Walisko, R., Melzer, G., Hempel, D.C., Kampen, I., Kwade, A., and Wittmann, C. (2013) Characterization and control of fungal morphology for improved production performance in biotechnology, *Journal of Biotechnology* 163, 112– 123.

Kusumaningtyas, E., Widiastuti, R., and Maryam, R. (2006) Reduction of aflatoxin B1 in chicken feed by using *Saccharomyces cerevisiae*, *Rhizopus oligosporus* and their combination, *Myopathology* 162, 307–311.

Kutter, S., Wille, G., Relle, S., Weiss, M. S., Hübner, G., and König, S. (2006) The crystal structure of pyruvate decarboxylase from *Kluyveromyces lactis*: Implications for the substrate activation mechanism of this enzyme, *Federation of European Biochemical Societies Journal* 273, 4199-4209.

Kutter, S., Spinka, M., Koch, M. H. J., and König, S. (2007) The influence of protein concentration on oligomer structure and catalytic function of two pyruvate decarboxylases, *The Protein Journal* 26, 585-591.

Leskovac, V., Trivic, S., and Anderson, B.M. (1998) Use of competitive dead-end inhibitors to determine the chemical mechanism of action of yeast alcohol dehydrogenase, *Molecular and cellular Biochemistry* 178, 219-227.

Lin, J.P., Ruan, S.D., and Cen, P.L. (1998) Mathematical model of L(+)-lactic acid fermentation in RDC coupled with product separation by ion exchange, *Chemical Engineering Communications* 168, 59-79.

Litchfield, J. H. (1996) Microbiological production of lactic acid, *Advance in Applied Microbiology* 42, 45–95.

Litchfield, J. H. (2009) Lactic Acid, microbially produced, *Applied Microbiology: Industrial* 362-372.

Lockwood, L. B., Ward, G. E., and May, O. E. (1936) The physiology of *Rhizopus oryzae*, *Journal of Agricultural Research* 53, 849–857.

Longacre, A., Reimers, J. M., Gannon, J. E., and Wright, B. E. (1997) Flux analysis of glucose metabolism in *Rhizopus oryzae* for the purpose of increasing lactate yields, *Fungal Genetics and Biology* 21, 30 – 39.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Rardall, A. J. (1951) Protein determination with the folin phenol reagent, *Journal of Biological Chemistry* 193, 265-275.

Lunt, J. (1998) Large-scale production, properties and commercial applications of polylactic acid polymers, *Polymer Degradation and Stability* 59, 145–152.

Ma, H., Zhang, S., Su, Z., and Wang, P. (2012) Kinetics and docking studies on the effect of chemical modification of NADH for redox reactions with dehydrogenase, *Journal of Molecular Catalysis B: Enzymatic,* 77, 111–118.

Martinez, F. A. C., Balciunas, E. M., Salgado, J. M., Domínguez, J. M., Converti, G. A., and Oliveira, R. P. D. S. (2013) Lactic acid properties, applications and production: A review, *Trends in Food Science and Technology* 30, 70-83.

Mcmartin, K. E., Sebastian, C. S., Dies, D., and Jacobsen D. (2012) Kinetics and metabolism of fomepizole in healthy humans, *Clinical Toxicology* 50, 375–383.

Miura, S., Arimura, T., Hoshino, M., Kojima, M., Dwiarti, L., and Okabe, M. (2003) Optimization and scale-up of l-lactic acid fermentation by mutant strain *Rhizopus sp*. MK-96-1196 in airlift bioreactors, *Journal of Bioscience and Bioengineering* 96, 65-69.

Moon, S. K., Wee, Y. J., and Choi, G. W. (2012) A novel lactic acid bacterium for the production of high purity L-lactic acid, *Lactobacillus paracasei* subsp. paracasei CHB2121, *Journal of Bioscience and Bioengineering* 114, 155-159.

Narayanan, N., Roychoudhury, P. K., and Srivastava, A. L. (2004) (+) lactic acid fermentation and its product polymerization, *Journal of Biotechnology* 7, 167-179.

Oda, Y., Yajima, Y., Kinoshita, M. Ohnishi, M. (2003) Differences of *Rhizopus oryzae* strains in organic acid synthesis and fatty acid composition, *Food Microbiology* 20, 371–375.

Papagianni, M. (2004) Fungal morphology and metabolite production in submerged mycelial processes, *Biotechnology Advances* 22, 189-259.

Park, E.Y., Kosakai, Y., and Okabe, M. (1998) Efficient production of L(+) -lactic acid using mycelial cotton-like flocs of *Rhizopus oryzae* in an air-lift bioreactor, *Biotechnology progress* 14, 699-704.

Pochanavanich, P., and Suntornsuk, W. (2002) Fungal chitosan production and its characterization, *Letters in Applied Microbiology* 35, 17–21.

Pritchard, G.G. (1971) An NAD^+-independent L-lactate dehydrogenase from *Rhizopus oryzae*, *Biochimica et Biophysica A*cta 250, 25-34.

Pritchard, G. G. (1973) Factors Affecting activity and synthesis of NAD^+-dependent lactate-dehydrogenase in *Rhizopus oryzae*, *Journal of General Microbiology* 78, 125-137.

Rosenberg, M., and Kristofikova, L. (1995) Physiological restriction of the L-lactic acid production by *Rhizopus arrhizus*, *Acta Biotechnologica* 15, 367–374.

Saito, K., Kawamura, Y., and Oda, Y. (2003) Role of the pectinolytic enzyme in the lactic acid fermentation of potato pulp by *Rhizopus oryzae*, *Journal of industrial Microbiology and Biotechnology* 30, 440–444.

Sande, M., and Monte, A. A. (2012) Fomepizole for severe disulfiram-ethanol reactions, *American Journal of Emergency Medicine* 30, 262.e3-262.e5.

Singlevich, T. E., and Barboriak, J. J. (1970) Pyrazole – induced inhibition of yeast alcohol dehydrogenase, *Biochemical Pharmacology* 20, 2087 – 2089.

Skory, C. D., Freer, S. N., and Bothast, R. J. (1998) Production of L-lactic acid by *Rhizopus oryzae* under oxygen limiting conditions, *Biotechnology Letter* 20, 191–194.

Skory, C. D., Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae*. *Applied and Environmental Microbiology* 66(2000): 2343–2348.

Skory, C. D. (2002) Homologous recombination and double-strand break repair in the transformation of *Rhizopus oryzae*, *Molecular Genetics and Genomics* 268, 397–406.

Skory, C.D. (2003) Induction of *Rhizopus oryzae* pyruvate decarboxylase genes, *Current Microbiology* 47, 59–64.

Skory, C. D. (2004) Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity, *Applied Microbiology and Biotechnology* 64, 237–242.

Skory, C. D., and Ibrahim, A.S. (2007) Native and modified lactate dehydrogenase expression in a fumaric acid producing isolate *Rhizopus oryzae* 99-880, *Current Genetics* 52, 23-33.

Skory, C. D., Hector, R. E., Gorsich, S.W., and Rich, J. O. (2010) Analysis of a functional lactate permease in the fungus *Rhizopus, Enzyme and Microbial Technology* 46, 43 – 50.

Smidt, O. D., Preeze, J. C. D., and Albertyn, J. (2008) The alcohol dehydrogenases *Saccharomyces cerevisiae*: a comprehensive review, *Federation of European Microbiological Societies* 8, 967–978.

Soccol C. R., Sonoga V. I., Raimbault M. (1994) Production of L-lactic acid by *Rhizopus* species, *World Journal of Microbiology and Biotechnology* 10, 433–435.

Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., and Van Wart, H. E. (1990) Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation, *Biochemistry. Proceedings of the National Academy of Sciences USA.* 87, 364–368.

Sun, Y., Li, Y. L., and Bai, S. (1999) Modeling of continuous L(+)-lactic acid production with immobilized *R. oryzae* in an airlift bioreactor, *Biochemical Engineering Journal* 3, 87–90.

Södergård, A., and Stolt, M. (2002) Properties of lactic acid based polymers and their correlation with composition, *Progress in Polymer Science* 27, 1123–1163.

Taber, R.L. (1998) The competitive inhibition of yeast alcohol dehydrogenase by 2,2,2-trifluoroethanol, *Biochemical Education* 26, 239-242.

Taherzadeh, M., Fox M., Hjorth H., and Edebo L. (2003) Production of mycelium biomass and ethanol from paper pulp sulfite liquor by *Rhizopus oryzae*, *Bioresource Technology* 88, 167-177.

Tay, A., and Yang, S.T. (2002) Production of L(+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor, *Biotechnology and Bioengineering* 80, 1–12.

Thitiprasert, S., Sooksai, S., and Thongchul, N. (2011) The in vivo regulation of alcohol dehydrogenase and lactate dehydrogenase in *Rhizopus oryzae* to improve L – lactic acid fermentation, *Applied Biochemistry and Biotechnology 164*, 1305 – 1322.

Thomas, G., Diefenbach, R., and Duggleby, R. G. (1990) Inactivation of pyruvate decarboxylase by 3-hydroxypyruvate, *Biochemical Journal* 266, 305-308.

Thongchul, N. (2005) Lactic acid production by immobilized *Rhizopus oryzae* in a rotating fibrous bed bioreactor. PhD thesis. The Ohio State University. Columbus, Ohio.

Thongchul, N., Navankasattusas, S., and Yang, S. T. (2010) Production of lactic acid and ethanol by *Rhizopus oryzae* integrated with cassava pulp hydrolysis, *Bioprocess and Biosystems Engineering* 33, 407–416.

Thongchul, N. (2013) Production of lactic acid and polylactic acid for industrial applications, In *Bioprocessing Technologies in Biofinery for Sustainable Production of Fuels, Chemicals, and Polymers* (S. T. Yang, H. A. El-Enshasy, & N. Thongchul Ed.) (John Wiley & Sons, Inc., New Jersey, 2013), pp 293–316 John Wiley & Sons, Inc., NJ.

Tobin, J. M., Cooper, D. G. and Neufeld, R. J. (1984) Uptake of metal ions by *Rhizopus arrhizus* biomass, *Applied and Environmental Microbiology* 47, 821–824.

Tsai, S. P., and Moon, S. H. (1998) An integrated bioconversion process for production of L(+)-lactic acid from starchy potato feedstocks, *Applied and Biochemical Biotechnology* 70-72, 417-428.

Umulis, D. M., Gurman, N. M., Singh, P., and Fogler, H. S. (2005) A physiologically based model for ethanol and acetaldehyde metabolism in human beings, *Alcohol* 35, 3 – 12.

Wang, J., Shen, N., Yin, H., Liu, C., Li, Y., and Li, Q. (2013) Development of industrial brewing yeast with low acetaldehyde production and improved flavor stability, *Applied Biochemistry and Biotechnology* 169, 1016–1025.

Wang, Q., Dajun, P. H., Shen, L. A., and Jiang, N. (2004) Purification, characterization, cloning and expression of pyruvate decarboxylase from *Torulopsis glabrata* IFO005, *The Journal of Biochemistry* 136, 447-455.

Wang, X., Sun, L., Wei, D., and Wang, R. (2005) Reducing by-product formation in L-lactic acid fermentation by *Rhizopus oryzae*, *Journal of Industrial Microbiology and Biotechnology* 32, 38–40.

Wang, Z., Wang, Y., Yang, S.-T., Wang, R., and Ren, H. (2010) A novel honeycomb matrix for cell immobilization to enhance lactic acid production by *Rhizopus oryzae*, *Bioresource Technolo*gy 101, 5557-5564.

Wee, Y. J., Kim, J.N., and Ryu, H. W. (2006) Biotechnological production of lactic acid and its recent applications, *Food Technology and Biotechnolgy* 44, 163-172.

Winslow, J. W. (1981) The reaction of sulfhydryl groups of sodium and potassium ion-activated adenosine triphosphatase with N-ethylmaleimide. The relationship between ligand-dependent alterations of nucleophilicity and enzymatic conformation states. *The Journal of Biological Chemistry* 256, 9522–9531.

Woiciechowski, A. L., Soccol, C. R., Ramos, L. P., and Pandey, A. (1999) Experimental design to enhance the production of L-(+)-lactic acid from steam-exploded wood hydrolysate using *Rhizopus oryzae* in a mixed-acid fermentation, *Process Biochemistry* 34, 949-955.

Wright, B. E., Longacre, A. and Reimers, J. (1996) Models of metabolism in *Rhizopus oryzae*, *Journal of Theoretical Biology* 182, 453–457.

Wu, X. F., Jiang, S. T., Liu, M., Pan, L. J., Zheng, Z., and Luo, S. Z. (2011) Production of l-lactic acid by *Rhizopus oryzae* using semicontinuous fermentation in bioreactor, *Journal of Industrial Microbiology and Biotechnology* 38, 565-571.

Yadav, A. K., Chaudhari, A. B., and Kothari, R. M. (2011) Bioconversion of renewable resources into lactic acid: an industrial view, *Critical Reviews in Biotechnology* 31, 1–19.

Yin, P. M., Nishina, N., Kosakai, Y., Yahiro, K., Park, Y., and Okabe, M. (1997) Enhanced production of L(−)-lactic acid from corn starch in a culture of *Rhizopus oryzae* using an air-lift bioreactor, *Journal of Fermentation and Bioengineering* 84 249–253.

Yoneya, T., and Sato, Y. (1979) Alcohol dehydrogenase from *Rhizopus javanicus*, *Applied and Environmental Microbiology* 37, 1073-1078.

Yu, M. C., Wang, R. C., Wang, C. Y., Duan, K. J., and Sheu, D. C. (2007) Enhanced production of L(+)-lactic acid by floc-form culture of *Rhizopus oryzae*, *Journal of the Chinese Institute of Chemical Engineers* 38, 223-228.

Yu, R. C. and Hang, Y. D. (1989) Kinetics of direct fermentation of agricultural commodities to L(+) lactic acid by *Rhizopus oryzae*, *Biotechnology Letters* 11, 597–600.

Yun, J. S., Wee, Y. J., and Ryu, H. W. (2003) Production of optically pure L(+)-lactic acid from various carbohydrates by batch fermentation of *Enterococcus faecalis* RKY1, *Enzyme and Microbial Technology* 33, 416–423.

Zhang, Z. Y., Jin, B., and Kelly, J. M. (2007) Production of lactic acid from renewable materials by *Rhizopus* fungi, *Biochemical Engineering Journal* 35, 251–263.

Zheng, S.Y., Xu, D., Wang, H. R., Li, J. and Zhou, H. M. (1997) Kinetics of irreversible inhibition of yeast alcohol dehydrogenase during modification by 4,4′-dithiodipyridine, *Journal of Biological Macromolecule* 20, 307-313.

Zhou, Y., Dominguez, J. M., Cao, N., Du, J. and Tsao, G. T. (1999) Optimization of L-lactic acid production from glucose by *Rhiopus oryzae* ATCC 52311, *Applied Biochemical and Biotechnology* 77-79, 401–407.

Zhou, Y., Dominguez, J. M., Cao, N., Du, J., and Tsao G. T. (1999) Optimization of L-lactic acid production from glucose by *Rhizopus oryzae* ATCC 52311, *Applied Biochemistry and Biotechnology* 77-79, 401-407.

Zhou, Y., Du J., and Tsao G. T. (2000) Mycelial pellet formation by *Rhizopus oryzae* ATCC 20344*, Applied Biochemistry and Biotechnology* 84–86, 779-789.

APPENDICES

APPENDIX A

SPORE MEASUREMENT AND PRODUCT DETERMINATION

1. Measurement of spore concentration

Sporangiospores were harvested from the surface of potato dextrose agar plat and extracting the spores with sterile water. To transfer 50 µL spores suspension onto the flat surface of hemacytometer and placing the glass cover slip on the top of the grids. Follow by placing the hemacytometer on the microscope and counting the total number of spores in each of the five squares at 40x power. Spores touching the top and left lines are counted which indicated below (fig. 1A).

Spore concentration calculation

A total volume of each square of the hemacytometer (25 squares) = 0.1 mm^3

The average number count of spores per square = X cells

The total cell number in 0.1 mm^3 = (X) × 25 cells

The total cell number in 1 mm^3 = (X) × 25× 10 cells

The toal cell number in 1 mL = (X) × 25× 10^4 cells/mL

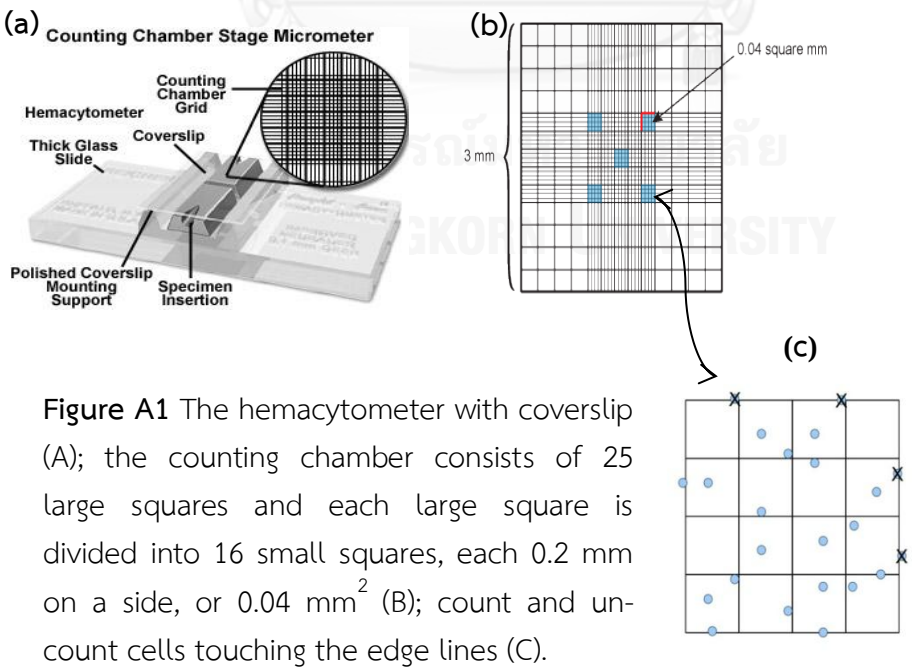
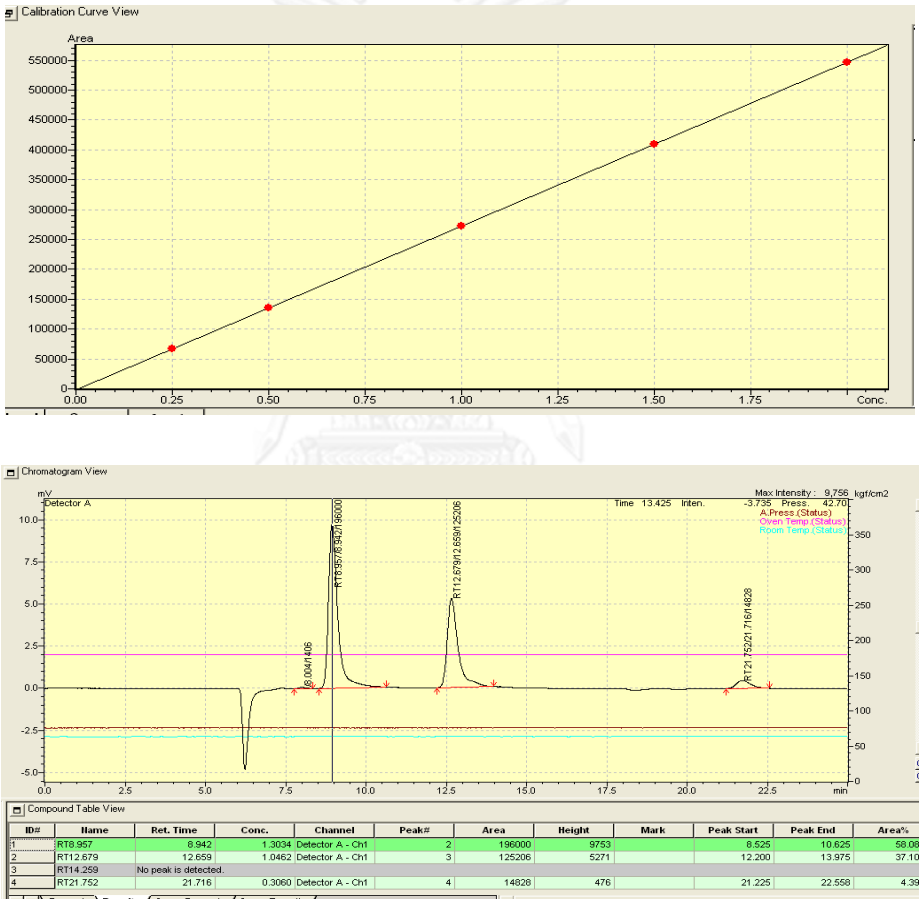


Figure A1 The hemacytometer with coverslip (A); the counting chamber consists of 25 large squares and each large square is divided into 16 small squares, each 0.2 mm on a side, or 0.04 mm^2 (B); count and un-count cells touching the edge lines (C).

2. Product determination

High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the fermentation broth. The samples from the fermentation broth were analyzed the concentration by diluting with distilled water and injected into an organic acid analysis column. A standard containing 0.25-2 g/L of each component were injected as a reference to determine the sample concentration (fig. 2.1A and 2.2A). The peak area was used for the comparison basis.



ID#	Name	Ret. Time	Conc.	Channel	Peak#	Area	Height	Mark	Peak Start	Peak End	Area%
1	RT8.957	8.942	1.3034	Detector A - Ch1	2	196000	9753		8.525	10.625	58.08
2	RT12.679	12.659	1.0462	Detector A - Ch1	3	125206	5271		12.200	13.975	37.10
3	RT14.259	No peak is detected.									
4	RT21.752	21.716	0.3060	Detector A - Ch1	4	14828	476		21.225	22.558	4.39

Figure A2 The standardize curve of lactic acid and sample HPLC chromatogram of lactic acid fermentation in glucose medium

3. Product yield ($Y_{p/s}$)

Product yield was determined from the ratio of product formed to glucose consumed during fermentation.

Example Control fermentation by immobilized *R. oryzae* in shake flask culture

Table A 1 Product yields in growth phase

Glucose (g/L)	Lactic acid (g/L)	Yield (g/g glucose)	Ethanol (g/L)	Yield (g/g glucose)
50.00	0.00	#DIV/0!	0.00	#DIV/0!
47.62	0.00	0.00	0.00	0.00
2.29	3.31	0.07	17.87	0.37
3.05	3.04	0.06	16.05	0.34
0.00	4.95	0.10	16.10	0.32
AVG		**0.08**		**0.35**

The lactic acid and ethanol yield in growth phase of 0.08 and 0.35 g/g glucose, respectively.

Table A 2 Product yield in production phase

Glucose (g/L)	Lactic acid (g/L)	Yield (g/g glucose)	Ethanol (g/L)	Yield (g/g glucose)
70.00	0.00	#DIV/0!	4.07	#DIV/0!
68.77	0.00	0	2.96	0
30.76	13.11	0.33	10.41	0.16
30.64	13.63	0.35	10.05	0.15
1.16	20.36	0.30	9.17	0.07
0.00	23.37	0.33	14.42	0.15
0.00	26.25	0.37	11.67	0.11
AVG		**0.34**		**0.13**

The lactic acid and ethanol yield in production phase of 0.34 and 0.13 g/g glucose, respectively.

4. Productivity

Volumetric productivity was defined as the total amount of product formed per volume per time.

Example Control fermentation by immobilized *R. oryzae* in shake flask culture

Table A 3 Volumetric productivities in growth phase

Time (h)	Lactic acid (g/L)	Productivity (g/L·h)	Ethanol (g/L)	Productivity (g/L·h)
0	0.00	#DIV/0!	0.00	#DIV/0!
6	0.00	0.00	0.00	0.00
24	3.31	0.14	17.87	0.74
30	3.04	0.10	16.05	0.54
48	4.95	0.10	16.10	0.34
AVG		**0.11**		**0.54**

The lactic acid and ethanol productivities in production phase of 0.11 and 0.54 g/L·h, respectively.

Table A 4 Volumetric productivities in production phase

Time (h)	Lactic acid (g/L)	Productivity (g/L·h)	Ethanol (g/L)	Productivity (g/L·h)
0	0.00	#DIV/0!	4.07	#DIV/0!
6	0.00	0	2.96	0
24	13.11	0.54	10.41	0.26
30	13.63	0.45	10.05	0.20
48	20.36	0.42	9.17	0.11
54	23.37	0.33	14.42	0.19
72	26.25	0.36	11.67	0.11
AVG		**0.42**		**0.19**

The lactic acid and ethanol yield in production phase of 0.42 and 0.19 g/ g/L·h, respectively.

APPENDIX B

APPARENT MAXIMUMENZYME REATION RATE (v_{max}) AND SUBSTRATE AFFINITY (K_m)

1. Lactate and ethanol production rate

During the production phase, the initial glucose concentration was varied from 10 to 90 g/L. Glucose, lactic acid, and ethanol concentrations were plotted at various times for rate determination (*v*). The slope of the plot were the production rate of lactic acid and ethanol.

Example Lactic acid and ethanol production rate in the fermentation by immobilized *R. oryzae* in production phase containing 70 g/L initial glucose concentration.

Table B1 Lactic acid and ethanol concentration during fermentation time of production phase

Time (h)	Lactic acid (g/L)	Ethanol (g/L)
0	0.00	0.00
8	0.00	0.00
16	1.62	0.00
24	6.24	0.00
32	10.03	1.88
40	20.09	6.04
48	20.73	6.49
56	23.38	6.30
64	26.15	7.50
72	25.52	7.82

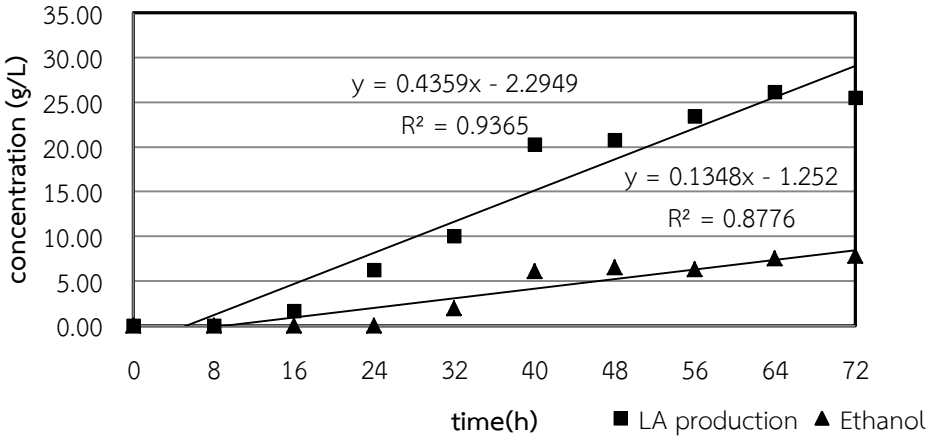


Figure B1 Lactic acid and ethanol production during production phase. Lactic acid and ethanol production rate were 0.44 and 0.13 g/L·h, respectively.

2. The apparent maximum enzyme reaction rate (v_{max}) and substrate affinity (K_m)

The apparent maximum reaction rate for lactate and ethanol production ($v_{max,app}$) and the affinity of glucose substrate for lactate and ethanol ($K_{m,app}$) were described by Michaelis-Menten kinetics. To obtain the Michaelis-Menten constants, modified Linweaver-Burk plots were used.

Example Lineweaver-Burk plots for determining $v_{max,app}$ and $K_{m,app}$ of lactate production in the fermentation by *R. oryzae* grown in the simple glucose medium (control)

Table B2 The reverse of glucose and lactic acid concentration

Glucose (g/L) $[S_0]$	$1/[S_0]$ (L/g)	Lactic acid production rate (g/L·h) (v)	$1/[v]$ (L·h/g)
10	0.100	0.13	7.42
20	0.050	0.26	3.78
30	0.033	0.30	3.37
50	0.020	0.42	2.40
70	0.014	0.41	2.45
90	0.011	0.57	1.75

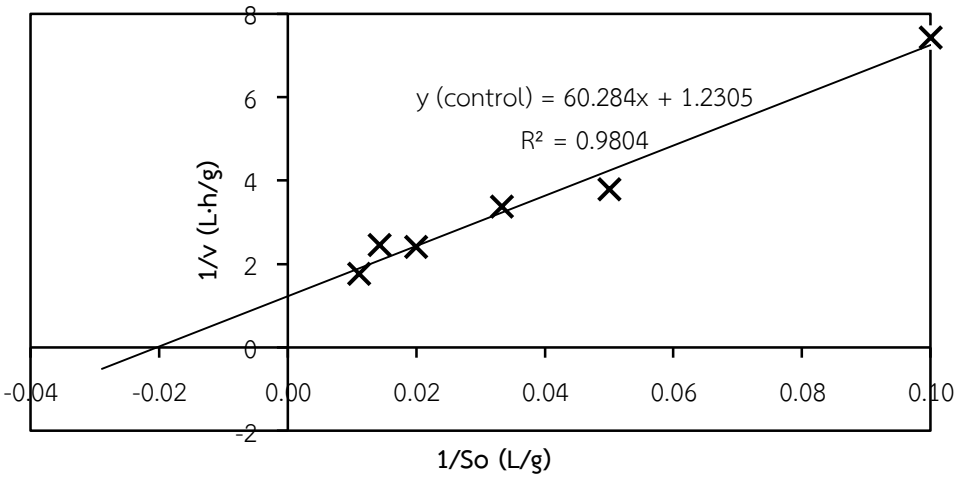


Figure B2 Lineweaver-Burk plot.

The $v_{max,app}$ and $K_{m,app}$ were calculated from Lineweaver-Burk plot equation indicated below

$$\frac{1}{v} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax}$$

From the modified Linweaver-Burk plot ;

Y-intercept $= \frac{1}{Vmax} = 1.2305$

Slope $= \frac{Km}{Vmax} = 60.284$

Hence, The $v_{max,app}$ and $K_{m,app}$ for lactate production were 0.81g/L·h and 48.99 g/L, respectively.

APPENDIX C

ENZYME ASSAY AND PROTEIN DETERMINATION

1. NAD^+-dependent LDH activity

LDH catalyzes the conversion of pyruvate into lactic acid by requiring NADH/NAD^+ as cofactor showed below :

$$\text{Pyruvate + NADH} \underset{\text{NAD}^+\text{-independent LDH}}{\overset{\text{NAD}^+\text{-dependent LDH}}{\rightleftharpoons}} \text{Lactic acid + NAD}^+$$

The activity of LDH was determined by the following the oxidation of NADH at absorbance 340 nm in the reaction mixture containing :

1. 2.50 mL 0.10 M Phosphate buffer (pH6.5)
2. 0.25 mL 0.10 M Sodium pyruvate
3. 0.10 mL 0.01 M NADH
4. 0.20 mL Mycelia extract
5. 0.10 mL Distilled water

The reaction was started with the reaction of the mycelia extract into the reaction mixture. LDH activity was detected for 7 min at 30 °C. The calibration plot was used for calculating NADH oxidized into micromole.

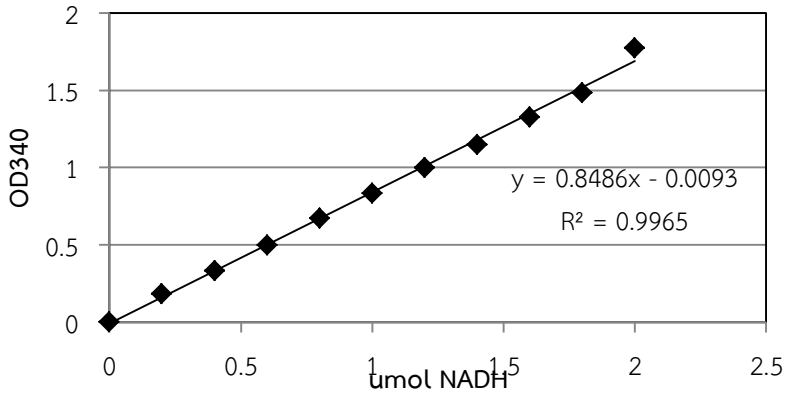


Figure C1 NADH standard curve for determining LDH activity

Specific activity was calculated by the oxidation of NADH in μmole under 1 min reaction time which was divided by the concentration of protein in mg/mL. Therefore, the specific activity value was quote as units/mg protein.

2. Alcohol dehydrogenase activities

2.1 Forward direction (ADH(f))

This enzyme converts acetaldehyde into ethanol by reducing NADH.

$$\text{Acetaldehyde} + \text{NADH} \xrightarrow{\text{ADH(f)}} \text{Ethanol} + \text{NAD}^+$$

The activity of ADH(f) was determined by the following the oxidation of NADH at absorbance 340 nm in the reaction mixture containing :

1. 2.50 mL 0.10 M Tris-HCl buffer (pH7.7)
2. 0.50 mL 5 M Acetaldehyde
3. 0.40 mL 0.01 M NADH
4. 0.20 mL Mycelial extract

The reaction was started with the same manner as that described in the LDH activity assay.

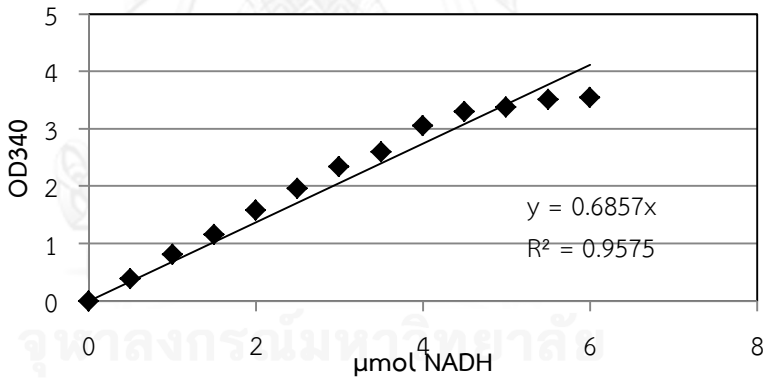


Figure C2 NADH standard curve for determining ADH(f) activity.

Specific activity was calculated by the oxidation of NADH in μmole under 1 min reaction time which was divided by the concentration of protein in mg/mL. Therefore, the specific activity value was quote as units/mg protein.

2.2 **Backward direction (ADH(b))**

This enzyme converts ethanol into acetaldehyde by oxidizing NAD^+.

$$\text{Ethanol} + NAD^+ \xrightarrow{\text{ADH(b)}} \text{Acetaldehyde} + NADH$$

The activity of ADH(b) was determined by the following the oxidation of NADH at absorbance 340 nm in the reaction mixture containing :

1. 2.50 mL 0.10 M Tris-HCl buffer (pH7.7)
2. 0.15 mL Absolute ethanol
3. 0.40 mL 0.01 M NAD^+
4. 0.20 mL Mycelia extract
5. 0.35 mL Distilled water

The reaction was started with the reaction of the mycelia extract into the reaction mixture. ADH(b) activity was detected for 7 min at 30 °C. The calibration plot was used for calculating NADH reduced into micromole.

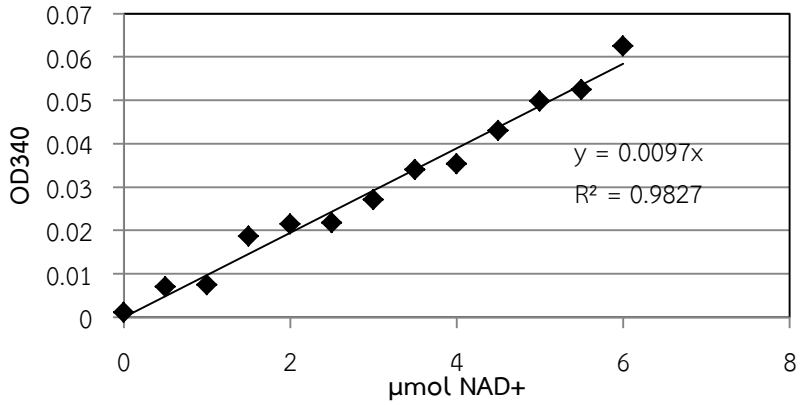
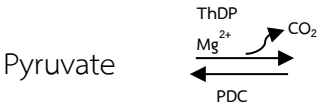


Figure C3 NADH standard curve for determining ADH(b) activity.

Specific activity was calculated by the oxidation of NADH in µmole under 1 min reaction time which was divided by the concentration of protein in mg/mL. Therefore, the specific activity value was quote as units/mg protein.

3. Pyruvate decarboxylase activity

PDC converts pyruvate into acetaldehyde by requiring TPP and $MgCl_2$ as the cofactors.



The activity of PDC was determined by the following the oxidation of NADH at absorbance 340 nm in the reaction mixture containing :

1. 0.30 mL 0.1 M MES buffer (pH6.5)
2. 0.60 mL 0.5 M Sodium pyruvate
3. 0.60 mL 0.01 M $MgCl_2$
4. 0.24 mL 0.01 M TPP
5. 0.264 mL 11 units ADH from baker yeast
6. 0.118 mL 0.01 M NADH
7. 0.20 mL Mycelial extract
5. 0.112 mL Distilled water

The reaction was initiated when the mycelial extract was added into the assay mixture. The change in absorbency at 340 nm for 7 min at 30 °C was monitored as the activity of PDC expressed in μM NADH oxidized per minute.

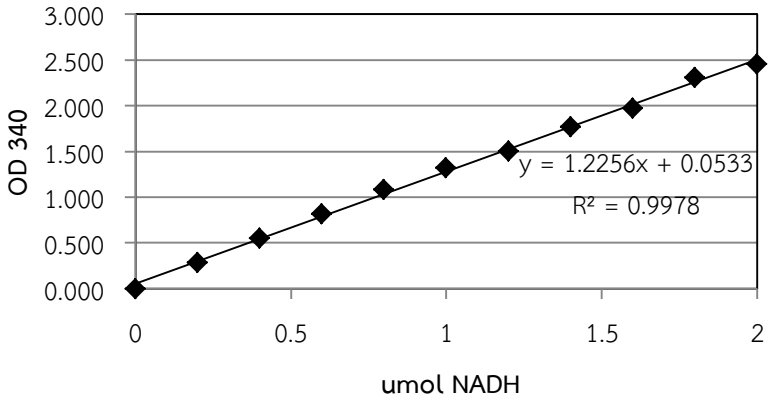


Figure C4 NADH standard curve for determining PDC activity**.**

Specific activity was calculated by the oxidation of NADH in μmole under 1 min reaction time which was divided by the concentration of protein in mg/mL. Therefore, the specific activity value was quote as units/mg protein.

4. Protein determination

Lowry method was used to determine the amount of protein present in the mycelial extract using bovine serum albumin as a standard. Two reagents were used for determining protein content, including :

Reagent A : 4 mL 2.0% sodium potassium tartrate
4 mL 1% $CuSO_4 \cdot 5H_2O$
392 mL 2.0% Na_2CO_3 in 0.1 N NaOH

Reagent B : A Folin phenol reagent

Both reagents were freshly prepared before use.

BSA standard curve was prepared by diluting 2 mg/mL BSA standard with 10-100 µL distilled water (0-200 µg/mL). Then 3 mL reagent A was added. The mixture was mixed thoroughly before incubation for 10 min. After that 0.3 mL reagent B was added into the mixture. The mixture was mixed well and incubated for another 30 min. After that the optical density of the mixture was observed at the wavelength of 650 nm.

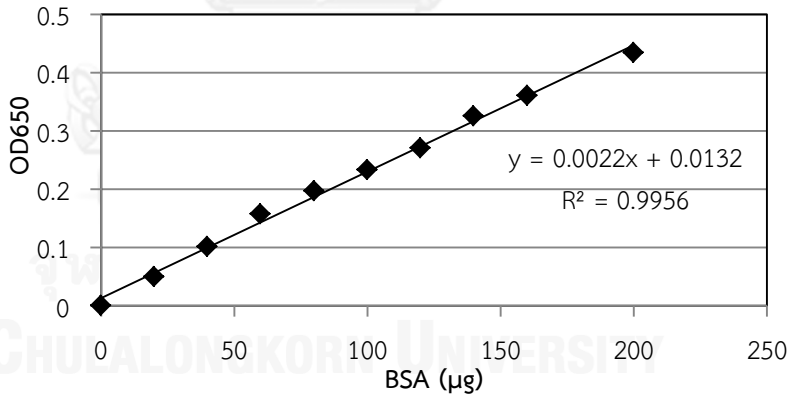


Figure C5 Bovine serum albumin standard for protein determination.

The protein content in the cell extract sample was determined by the same method used in standard curve preparation. 3 mL reagent A was mixed with 0.1 mL cell extract. The mixture was incubated for 10 min. After incubation, 0.3 mL reagent B was added into the mixture. After mixing thoroughly, the mixture was incubated for 30 min. The optical density of the mixture was measured at the wavelength of 650

nm and the protein concentration in the sample was calculated from the BSA standard curve.

VITA

Miss Sitanan Thitiprasert was born on July 10, 1992 in Nakhon Si Thammarat, Thailand. She graduated with Bachelor's Degree of Science in Fermentation Technology, from King Mongkut's Institute of Technology Ladkrabang. Later, she worked at the Institute of Biotechnology and Genetic engineering, Chulalongkorn University as a researcher for 2 years. In 2007, she began her Master's degree in Biotechnology, Faculty of Science, Chulalongkorn University. Two years later, she has continued her study to Doctoral degree in the same program as Master's degree. During her studies, she was supported by Thailand Research Fund and the Graduate School of Chulalongkorn University via the TRF Master Research Grant (MRG-WII51E053) and 90th anniversary of Chulalongkorn University. In 2011, she was awarded the technology grants Southeast Asia from OeAD scholarship for having an experience at Graz University of Technology, Austria for a year.