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OPIMIZATION OF ITACONIC ACID FERMENTATION IN A SHAKEN FLASK AND PRODUCTIO N SCALING UP

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บงกช วกุลชัย : การหาภาวะที่เหมาะสมในการหมักกรดอิทาโคนิคในขวดเขย่าและขยายส่วนการผลิต (OPIMIZATION OF ITACONIC ACID FERMENTATION IN A SHAKEN FLASK AND PRODUCTION SCALING UP) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ณัฏฐา ทองจุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.เคนทาโร โคดามะ, หน้า.

ภาวะต่างๆของการผลิตกรดอิทาโคนิคโดย *Aspergillus terreus* ถูกศึกษาเพื่อการผลิตกรดอิทาโคนิค โดยในกระบวนการหมักได้แบ่งออกเป็นสองช่วงคือ ช่วงการเจริญเติบโตของเซลล์ และการผลิตกรดอิทาโคนิค ในช่วงของการเจริญเติบโตของเซลล์นั้น อาหารเลี้ยงเชื้อที่เหมาะสมต่อการเจริญของเซลล์คือ อาหารจำพวกสารอินทรีย์ โดยสูตรอาหารเลี้ยงเชื้อที่เหมาะสมต่อการเจริญเติบโตของเซลล์ประกอบไปด้วย น้ำตาลกลูโคส และ สารสกัดจากยีสต์ซึ่งให้ผลผลิตของเซลล์มากที่สุดหลังจากทำการหมักเป็นเวลา 3 วัน ในขณะที่การหมักเพื่อผลิตกรดอิทาโคนิคนั้น เป็นสารอาหารจำพวกสารประกอบอนินทรีย์ที่ให้ผลผลิตของกรดอิทาโคนิคมากที่สุด ในการทดลองระดับขวดเขย่า พบว่า มีปริมาณความเข้มข้นของกรดอิทาโคนิคถูกปลดปล่อยออกมาสูงถึง 21.98 กรัมต่อลิตร ภายใต้ภาวะ 30 องศาเซลเซียส ค่าความเป็นกรดด่างที่2 และเขย่าที่ความเร็วรอบ 200 รอบต่อนาที เป็นเวลา 7 วัน นอกจากนี้ยังพบว่า ลักษณะสัณฐานที่เหมาะสมต่อการผลิตกรดอิทาโคนิคคือเม็ดทรงกลมขนาดเล็ก ในขณะที่การผลิตกรดอิทาโคนิคในระดับที่ใหญ่ขึ้น ไม่เป็นผลสำเร็จนัก ในถังหมักระดับ 5 ลิตร พบว่า มีปริมาณกรดอิทาโคนิคเพียงเล็กน้อยเท่านั้นถูกปลดปล่อยออกมาจากการหมักของ *A. terreus* เมื่อทำการเพิ่มความเร็วในการกวน พบว่าปริมาณการผลิตกรดอิทาโคนิคไม่เพิ่มขึ้น แต่พบการบริโภคน้ำตาลกลูโคสของเซลล์ อย่างไรก็ตาม เซลล์ไม่ได้นำการสลายของน้ำตาลกลูโคสไปใช้เพื่อผลิตกรดอิทาโคนิค แต่ใช้เพื่อการเจริญเติบโตของเซลล์และสร้างพลังงาน ATP เพื่อความอยู่รอดของเซลล์ นอกจากนี้ ในภาวะดังกล่าวนี้ ไม่พบลักษณะสัณฐานซึ่งเป็นเม็ดทรงกลมขนาดเล็กที่เหมาะสมต่อการผลิตกรดอิทาโคนิค ในงานวิจัยชิ้นนี้ได้ทำการตรึงด้วยเทคนิค entrapment อีกด้วย โดยใช้แคลเซียมอัลจิเนตเป็นวัสดุตรึง พบว่ามีการปรากฏสัณฐานทรงกลมขนาดเล็กระหว่างการหมัก แต่อย่างไรก็ตาม ปริมาณการผลิตกรดอิทาโคนิคยังคงน้อยเมื่อเทียบกับการหมักในระดับขวดเขย่า ทั้งนี้อาจจะมาจากวัสดุที่ใช้ในการตรึงไม่เหมาะสมต่อการผลิตกรดอิทาโคนิค และประสิทธิภาพการผลิตกรดอิทาโคนิค ยังคงขึ้นอยู่กับปัจจัยหลายๆด้าย อาทิเช่น ธรรมชาติของเซลล์ อาหารเลี้ยงเชื้ออีกด้วยเช่นกัน

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The different fermentation condition of itaconic acid production by *Aspergillus terreus* was studied. The fermentation was divided into two phases: cell growth phase and itaconic acid production phase. In cell growth phase, the organic medium is preferred for cell growth. The highest cell biomass was reached after 3 days with glucose - yeast extract medium. Whereas itaconic production phase, the inorganic medium was lead to the maximum itaconic acid concentration. In flasks shaken, it was found that there is 21.98 g/L of itaconic acid were released at 30°C, pH 2 and 200 rpm for 7 days. Moreover, the morphology of *A.terreus* that suit itaconic acid production is a small pellet was found in this condition. When the production was scaled up, there is not successfully for itaconic acid production. In 5 L of stirred tank reactor, there is a little bit of itaconic acid were reached. Despite agitation speed were increased, itaconic acid production is not increase. However, glucose was consumed for cell growth and generating ATP for survival not itaconic acid formation. In addition, a small pellet which suit for itaconic acid production was not found in 5L bioreactor. Immobilization technique including adsorption and entrapment were also tested for itaconic acid production but with a poor result. It cannot compete with the production from a small scale like flasks shaken. However, the performance of cell immobilized might be depending on a nature of cell, medium composition and other factors.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CONTENTS

Page

Page

Page

LIST OF TABLE

LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1 Background

Itaconic acid is an unsaturated carboxylic acid. It's used for synthesis of resins, plastics, fibers, and rubbers. It can co-operated into polymers and may replace petrochemical-based such as methacrylic acid. In addition, itaconic acid was non-toxic and good for environment. That's why Itaconic acid has gained increasing interest. Itaconic acid was first discovered by Baup in 1837 via the distillation of citric acid. Later, in 1931 itaconic acid was discovered again by Kinoshita via fungal fermentation and *Aspergillus itaconicus* was the first producer. But anyway the chemical synthesis cannot compete with the biosynthesis by fungi. That mean the biosynthesis was very well-known for itaconic acid production. Then, a researcher found that *Aspergillus terreus* can produce itaconic acid and it can give a higher yield. Up till now, *A.terreus* is most frequently used as a commercial producer of itaconic acid

Metabolism pathway of itaconic acid production via microorganism was not yet fully understood. There are three pathways that referred to itaconic acid production by biosynthesis. Especially, the first pathway seem most possible. The main route is via glycolysis pathway and tricarboxylic acid cycle [1] In glycolysis pathway, glucose is converted to pyruvic acid and then to acetyl co-enzyme A and enters the tricarboxylic acid cycle or TCA cycle. In TCA cycle, citric acid and cis-aconitric acid are intermediates. Many chemical reaction was found in TCA cycle like citric acid dehydration reaction. Finally, itaconic acid was formed from aconitate decarboxylase. The second pathway that referred is convert 1,2,3-tricarboxypropen to itaconic acid [2]. The last suggested pathway considered the condensation of pyruvate and acetyl Co-enzyme A to citramalate [3]. However, the second and third not well-known for itaconic acid production by biosynthesis.

In order to in crease the production rate of itaconic acid. There are many factor that should be concerned. The first one is medium composition such as carbon and nitrogen. The previous study was reported that carbon source have influence on itaconic acid production. When sucrose was used as carbon source the

production rate of itaconic acid is higher than used glucose as carbon source [4], [5]. Not only the carbon source but the concentration of carbon also effective on itaconic acid production. It was found that 5-7%(W/V) of glucose was preference. Nitrogen source also effective on production of itaconic acid. A researcher found that nitrogen source from ammonium sulfate give a high production rate of itaconic acid whereas other nitrogen source give a low production of itaconic acid [6]. However, the concentration of nitrogen should be low to prevent cell growth instead produce itaconic acid. If the concentration is very low or not enough, cell can not be alive and not found itaconic acid in this case [7]. The next chapter that should be concern is pH. pH should be keep low to prevent other organic acid or by-product form from TCA cycle[8]. Between 1.8-2.1 is preferable for itaconic acid production because it is optimum pH for enzyme activities in itaconic acid pathway [9] [4] [1]. Itaconic acid production was not presence when pH value is higher than 6. Last but not least, the temperature have effected on itaconic acid production. 30 degree Celsius of temperature is prefer for itaconic acid production. The last factor is oxygen, it needed for itaconic acid production and cell growth especially filamentous fungi.

There have been researchers regarding the itaconic acid production from *A.terreus*. For example, In 1945 Lockwood and Ward found that *A.terreus* NRRL 1960 reach 49.9g itaconic acid from 165g/L of glucose 2.5g/L ammonium nitrate and 4mg/L of corn steep liquor and cell was performed at 30 degree Celsius 5L/min of air flow rate for 7 days. Nelson et al. reported that 45-54%yield of itaconic acid can be achieved with *A.terreus* from 60 g/L of initial glucose 2.67g/L of ammonium sulfate and 1.5g/L of corn steep liquor after 4-6 days using 0.8% (V/V) inoculum and performed at pH 1.8-2 in 20L fermenters with agitation speed about 100 rpm. In 1945, Eimhjellan and Larsen investigated the ability of *A.terreus* to form itaconic acid from variety of carbon source 100g/L of glucose sucrose and cellobios were also used as carbon source and they found sucrose reach 57 g itaconic acid and glucose and cellobios reached 52 and 41 g itaconic acid, respectively. Later, in 1975 Elnaghy and Megalla investigated effect of temperature and nitrogen source. They found at 30 ℃ and pH3.5 were optimum for itaconic acid production. Moreover, they suggested that the most suitable nitrogen source for itaconic acid production was peptone [5]. Riscaldati et al. investigated effect of pH and stirring rate on itaconic

acid production by *A.terreus*. *A.terreus* were performed by varying the pH in the range 1.85 – 2.8 under a constant stirring rate of 320 rpm. It was found that at pH 2.4 and 320 rpm gave a highest yield of itaconic acid. It was 0.53 g itaconic acid/g substrate. In addition, strain improvement of *A.terreus* by mutagenesis were also investigated [10]. Yahiro et al. reported that the mutant strain TN-484 can produce more than 65 g/L of itaconic acid. It was selected as the most promising high itaconic acid yield producer [11]. In 2007 Dwiarti et al. reported that *A.terreus* TN 484-M1 reached 0.34 g itaconic acid/ g substrate when sago starch hydrolysate used as carbon source [12]. In 2012 Kuenz et al. reported the itaconic acid concentration of 86.2 g/L was achieved within 7 days when performed in 10L stir tank reactor and 180 g/L of glucose used as carbon source [13].

Nevertheless, the yield of itaconic acid was still low. This somewhat makes itaconic acid cost uncompetitive to petroleum based feedstock. This is a motivation of this research. The aim of this study is to optimize the cultivation conditions to promote itaconic acid synthesis from glucose based medium by *A.terreus* NRRL1960.

1.2 Objective and scope of research

The aim of this study was improve the itaconic acid production by fermentation of *A.terreus* NRRL 1960. The scope of this work was including physical factor like pH, rotation speed, agitation speed, morphology and chemical factor like medium composition for itaconic acid production. It believed that the results in this study will be helpful for developing the industrial itaconic acid production by fermentation of *A.terreus*

CHAPTER 2

THEORETICAL AND LITERATURE REVIEW

2.1 Properties and Applications

Itaconic acid (IA) is an unsaturated dicarboxylic organic acid also known as methylene succinic acid. IA has the stoichiometric formula $C_5H_6O_4$ and is composed with one methylene group and two carboxylic groups. In solid state, IA is white crystalline with the molecular weight of 130.1 g/mol, density of 1.573 g/L, a melting point of 165-168 °C and dissolves in water up to 80.1 g/L at 20 °C. IA can also dissolve in alcohols including methanol, 2-propanol and ethanol. The acid is stable at acidic, neutral and middle basic conditions at moderate temperature [14]. It has two pKa values, one at 3.85 and the other at 5.45. The degree of reduction is 3.6; it is more oxidized than glucose. It can be separated using ion-exchange chromatography and detected via UV-spectroscopy at a wavelength of 210 nm or RI detectors [15].

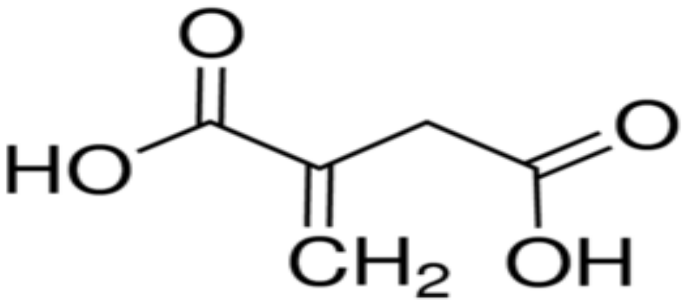


Figure 2. 1 Structure of itaconic acid

Figure 2. 2 Itaconic acid

IA is one of the most promising substances within the group of organic acids [16]. and its flexible building blocks are derived from biomass [17]. IA is readily biodegradable in nature. IA can substitute methacylic acid, which is currently

provided by the petrochemical industry [18]. It can either be self-polymerised to polyitaconic acid or can act as a monomer with other monomers to form a heteropolymer, such as acrylamide [16]. The several functional and unique properties of IA has led to increasing interest surrounding it as a future bio-based platform chemical. For instance, it can replace petrol-based compounds such as methacrylic acid in industry [19]. Recently, it was shown that IA can act as a precursor for several potential biofuels such as 3-mwthyltetrahydrofuran (3-MTHF) [20] and has been successfully applied as a co-polymer together with several other chemicals. IA also has several industrial applications including additives, carbon fiber technology, resin coating and rubber. Moreover, it can be used in hair treatment and the influence of IA on the wet scrub resistance of high pigment paints for architectural coating [21].

IA has further diverse applications. Although used in many industries, it is also sometimes an end product. Common end products of IA polymerization such as polyitaconic acid (PIA) can replace the usage of sodium tripolyphosphate (STPP) in

the detergents industry. Another end product of IA polymerization is styrene-butadiene rubber (SBR), which is made from the polymerization of styrene, butadiene and itaconic acid. When 1-5% of IA is added in SRB, it exhibits a superior adhesion and is preferred for use in carpet backing and paper coatings. For the industrial usages of resins, lattices and fibres, the polymerized methyl, ethyl and vinyl esters of IA are used as plastic, elastomer adhesives and coating. Additionally, copolymers of IA yield rubber-like resin of excellent strength and flexibility and waterproofing coating with good electrical insulation [16]. Itaconic anhydride can be used in the preparation of monoesters like monomethyl itaconate and also reacts with amine to yield N-substitute pyrrolidones. Another application of IA is in the shampoo and detergent industries. Alkali salts of the homopolymer poly IA have been recommended for use in detergents[16]. In the detergents industry, IA further competes with fumaric and malic acid [22]. The acid is also used in the shampoo industry with the imidazole derivative – an active ingredient in shampoos, condensated by luric acid and the aminoethylethanolamine reacts with itaconic acid [7]. For the emulsion paints industry, IA can improve the adhesion of the polymer.

When 5% of IA is added to acrylic resin, it increases the ability of the resin to hold printing inks [23].IA can the use of replace arylic acid in the production of superabsorbent polymers for improved properties. For plastics and coating manufacturing, a 1-5% addition of IA produces product benefits such as a light colour, case of printing and separation, and antiseptic properties [23]. IA is also utilised in the medical and pharmaceutical fields, such as in dentistry and contact lenses. For the former it is used in the production of glass ionomer cement (GIC) biocompatible cemen,t while in the latter as a hardening agent in organ osiloxanes (El-lman and Du, 2014). In addition, IA is applied in water treatment technology. Recently, it was shown that IA can act as a precursor for several potential biofuels such as 3-mwthyltetrahydrofuran (3-MTHF) [20].

2.2 History of itaconic acid

IA was first discovered by Baup (1837) after being formed by the distillation of citric acid. This was the first method and the main process for IA production. Later, it was produced using various chemical methods. Decarboxylation of aconitic acid is one chemical process that provides itaconic acid [24]. Another is the patented

montecatini method involving propargyl chloride [25]. Another chemical method, devised by Berg and Hetzel, involved the oxidation of mesityl oxide and the subsequent isomerization of the formed citric acid [26] . Additionally, itaconic acid has also been formed via chemical synthesis thoguh the oxidation of isoprene [27].

Not only do chemical processes produce itaconic acid, but also the fermentation process by microorganisms. Significantly, itaconic acid was formed again by Kinoshita [28]. The acid was formed during the growth of *A.itaconicous* on acid medium. This strain was the first producer of itaconic acid. Later, Calm et al. reported that *A.terreus* can also release and yield greater amounts of itaconic acid than itaconic acid from *A.itaconicous*. *A.terreus* is thus more suitable and has subsequently become a commercial strain. The fungus has become widely known as an excellent biological producer of itaconic acid.

2.3 Microorganisms

Lockwood and Reeves (1945) screened more than 300 identified wild type strains for the Northern Regional Research Laboratory (NRRL) of the U.S. Department

of Agriculture in Peoria, Illinois. It was found that *A.terreus* NRRL1960 was identified as the most prolific itaconic acid production strain [29]. Finally, *A.terreus* NRRL1960 become the most public strain [16]. In 1995, Gyamerah reported that *A. terreus* NRRL1960 could release 55 g/L of itaconic acid within 8 days [30]. In 1962, Nubel and Ratajak reported that *A.terreus* NRRL 1960 could produce 70.8 g/L of itaconic acid within 3 days[31]. Further to this, Batti and Schweiger (1963) also reported the production of 89.6 g/L of itaconic acid during the fermentation of *A.terreus* NRRL 1960 within 7.7 days. Other strains of *A.terreus* can also form itaconic acid. *A.terreus* DM-23081 can reach 86.2 g/L of itaconic acid within 7 days from a glucose medium [13]. *A.terreus* IMI 282743 can release 5.76 g/L of itaconic acid from palm oil [32] whereas a local strain of *A.terreus* can reach 54 g/L of itaconic acid [5].

Even though the *A.terreus* strain is the best producer of itaconic acid, researchers have also identified other microorganisms that can produce itaconic acid. To avoid some problems from filamentous fungi such as morphology control, shear stress and improved process of itaconic acid fermentation. Several strains have been

tested for itaconic acid production. This notably includes *Candida sp*., of which 35 g/L of itaconic acid was obtained from glucose medium within 5 days [33]. A *Candida* mutant was able to release 42 g/L of itaconic acid within 5 days [34]. *Ustilago sp.* was also tested by Iwata Corp.(Japan) and found that *U maydis* can produce 53 g/L of itaconic acid from glucose medium after 5 days [33], while *Rhodotarula sp* can produce 15 g/L of itaconic acid from glucose medium after 7 days [35]. However, other microorganisms still produce small amounts of itaconic acid when compared with *A.terreus*. It has been clearly shown that none of these other microorganisms can compete with the fermentation from *A.terreus* strain.

2.3.1 Strain improvement by genetic engineering

To improve itaconic acid production, a mutant strain of *A.terreus* was also tested for itaconic acid production. To avoid the problem of product inhibition, *A.terreus* IFO-6365 was used as the parent strain for the selection of mutant strains with NTG-treatment. The improved strain; *A.terreus* TN484 can release 82 g/L of itaconic acid from glucose media after 6 days [11]. Further to this, *A.terreus* TN484 was tested again with raw cornstarch medium with 60 g/L of itaconic acid produced

in a flask culture [36]. Another strain of *A.terreus* was also tested after being treated via mutagenesis with *A.terreus* SKR10 the parent strain. After using ultraviolet mutagens to separate this strain, there are two improved strains. One is *A.terreus* N50, which can reach 50 g/L of itaconic acid from corn starch. The other is *A.terreus* UNCS1, which releases 32 g/L of itaconic acid from waste extracts. The parent strain *A.terreus* SKR10 itself can produce 32 g/L of itaconic acid from corn starch and 20 g/L of itaconic acid from fruit waste extracts. It cannot compete with the mutant strains [37] . *A.terreus* TN484 M1 – a mutant strain from *A.terreus* NRRL1960 – can reach 80 g/L of itaconic acid within 6 days [12]. Recently, genetic engineering was used to enhance itaconic acid production. *A.terreus* was modified by incorporating *A.niger*. Following insertion of a modified pfkA gene from *A.niger* into *A.terreus*, it was found that a mutant strain could reach a higher itaconic acid concentration than a parent strain. Indeed, the best mutant strain produces 45.5 g/L itaconic acid, whereas the wild type strain can release only 21.35 g/L of itaconic acid [38]. In 2014, Huang et al. reported that itaconic acid production can be improved through the genetic engineering of an industrial *A.terreus* strain. The gene is closely related to itaconic

acid production including cadA and mfsA and was identified and expressed in *A.terreus* strain. It was found that overexpression of the gene cadA (cis-aconitate decarboxylase) led to an increase in itaconic acid production levels by 9.4% while overexpression of mfsA achieved an itaconic acid production level of 5.1%.

2.4 Pathway and key enzymes for itaconic acid production

In 1931, itaconic acid was discovered by Kinoshita who discussed the decarboxylation of aconitate as a key reaction that leads to itaconic acid formation. Later, Bentley and Thissen (1957) showed a strong indication for the presence of cis-aconitate decarboxylase (CAD) in the cell of *A.terreus* that might catalyze the reaction into itaconic acid. Although other researchers have tried to describe alternative pathways for itaconic acid production, there are three identified pathways from pyruvate to itaonic acid that have been discussed over previous decades.

1. In the first pathway, pyruvate is incorporated into the tricarboxylic acid cycle (TCA) and itaconic acid is formed by the decarboxylation of cis-aconitate decarboxylase [28].

2. The second pathway concerns the condensation of pyruvate and acetyl CoA to citramalate [3].
3. The third pathway, formation of 1,2,3–tricarboxypropanoate undergoes a dehydrate generation reaction to yield aconitric acid and, subsequently, itaconic acid [2].

However, it seems most likely that, according to Kinoshita (1931) the first pathway is the main one. The main route is via the glycolysis pathway. Glucose is broken down to create two molecules of pyruvate. Next, the pyruvate is converted to acetyl– CoA and enters the TCA cycle. In mitochondria, acetyl CoA is converted to citrate and to the cis-aconitate. Normally, cis-aconitate is converted to isocitrate and to the succinate and finally to the oxaloacetate. This is the complete TCA cycle. However, itaconic acid formation is not like this TCA cycle when cis-aconitate is released. Cis-aconitate is the intermediate for itaconic acid formation, and it is transferred to cytosol. Finally, itaconate is formed from cis-aconitate by cis-aconitate decarboxylase (CAD) and itaconate secreted from cytosol in itaconic acid form.

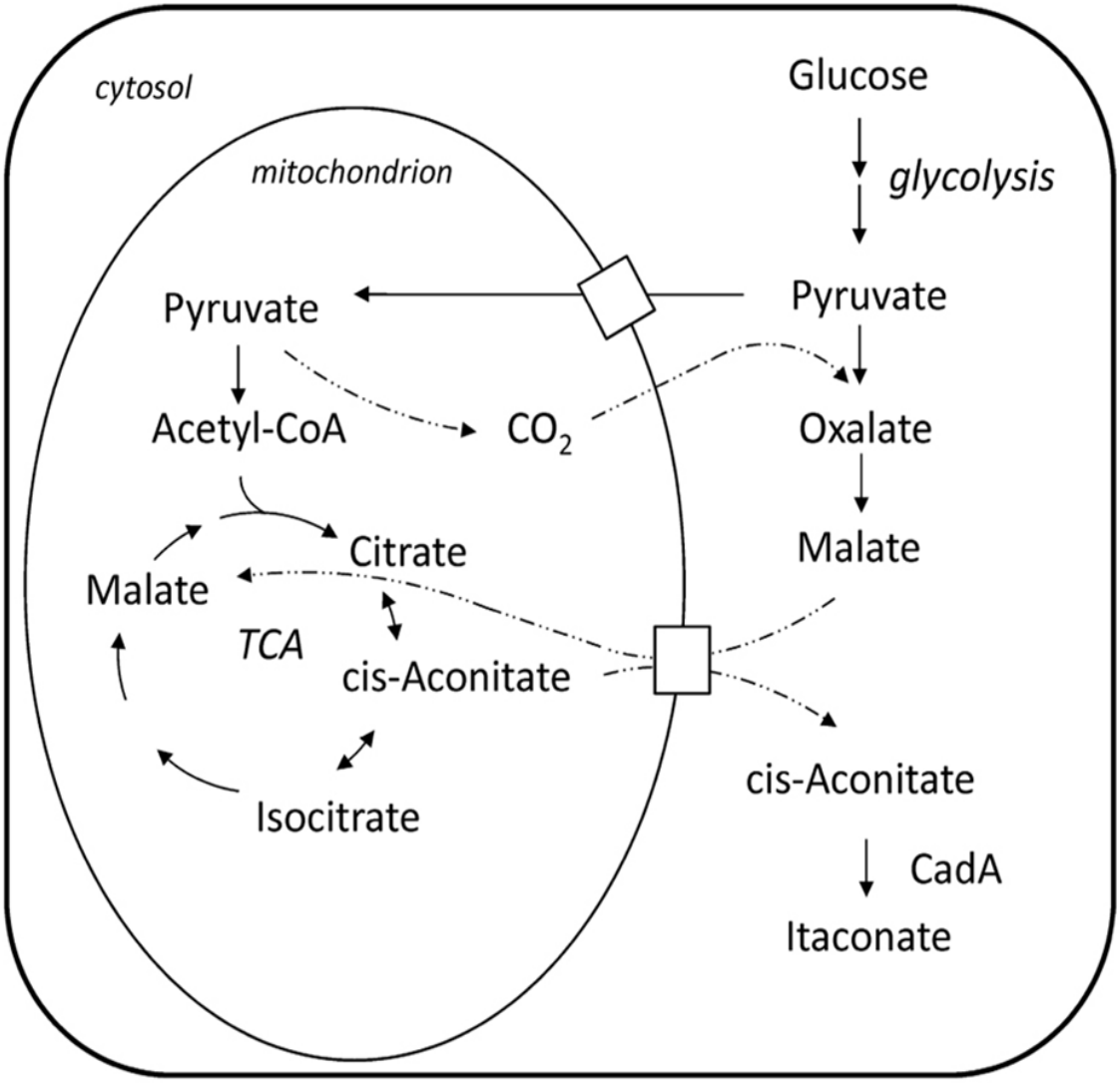


Figure 2. 3 Metabolic pathway of itaconic acid

Cis-aconitate decarboxylase (CAD) is one of the key enzymes for IA production by *A.terreus*. It is located at the cytosol where the IA is formed [39]. Cis-aconitate is converted to IA by the catalysis of cis-aconitate decarboxylase. This step

was confirmed by tracer studies with ^{14}C and ^{13}C substrates [1]. The next key enzyme is Aconitase. Before IA is formed, cis-aconitate is synthesized by this key enzyme. Aconitase converts citrate to cis-aconitate which is a precursor for IA production. Cis-aconitate is found in mitochondria and transported to the cytosol. Normally, there are two catalytic reactions involving aconitase: one converts citrate to cis-aconitate, while the other converts cis-aconitate to isocitrate. For IA production, it was found that more than 90% of the equilibrium of these reactions lies at the side of citrate. In addition, no citric acid can be found in IA production by *A.terreus* culture. This means that almost all the citrate is converted to cis-aconitate for IA production. Cis-aconitate is quickly removed from mitochondria to cytosol for eliminating the potential feedback inhibition of PFK by citrate itself [19]. Additionally, the level of aconitase and its activity are increased under IA production conditions by *A. terreus* [39]. Phosphofructokinase (PFK) is another enzyme related with IA production. In the glycolysis pathway, glucose breaks down pyruvate through the activities of PFK. Then, pyruvate is converted to acetyl CoA for citrate formation by citrate synthase. However, PFK is inhibited by high levels of energy and citrate. Therefore, IA

production requires a decrease in the ATP level for accelerating the glycolysis pathway leading to the generation of new pyruvate, citrate and finally IA. TCA is transported by the malate/citrate antiport, and this antiport has a higher affinity for cis-aconitate than for citrate for significant cis-aconitate transport to occur [39].

2.5 Fermentation process

2.5.1 Medium composition

An excess carbon source, limited nitrogen source and low phosphate are required for TCA acid including itaconic acid. Glucose is broken down and utilized via glycolytic flux for the activity of cells such as cell growth and product formation. However, the C/N ratio should be high to enhance the accumulation and overproduction of TCA acids. The phosphate level should be low to prevent cell growth.

Carbon sources can be obtained from glucose, sucrose, starch, and molasses and hydrolyses of corn syrup. The best yield of itaconic acid is obtained by glucose and sucrose. For the *A.terreus* strain, glucose is commonly used as a carbon source for itaconic acid production. The effect of the carbon source on itaconic acid

production by *A.terreus* NRRL 1960 has been tested. Glucose, sucrose and cellobios have all been tried, and it was found that the highest yield of itaconic acid was obtained when sucrose was used as the carbon source – 57g/L of itaconic acid was released and 100 g/L of glucose consumed for acid formation [4]. Nubel and Ratajak reported that 50 weight % yield of itaconic acid was achieved when beet and cane molasses were used as carbon substrates [31]. The *A.terreus* strain, including DSM23081, NRRL1960 and NRRL1963, can release itaconic acid concentrations of up to 87-91 g/L of itaconic acid from glucose medium after 7 days [13]. Sucrose can also be used as the carbon substrate for itaconic acid production. In a sucrose medium 59 g/L of itaconic acid was obtained from *A.terreus* within 3 days [29]. In contrast, starch hydrolysate can reach 55 g/L of itaconic acid by fermentation of *A.terreus* M8 within 4 days [40]. *A.terreus* SKR10 reached 48.2 g/L of itaconic acid from sago starch medium after 6 days [12].

Additionally, nitrogen sources have also been considered for itaconic acid production. Both organic and inorganic compounds can be used as nitrogen sources

for acid formation. In the case of an inorganic compound, ammonium sulfate and ammonium nitrate were investigated for itaconic acid formation. However, ammonium sulfate is better than ammonium nitrate as it has led to the highest yield of itaconic acid by fermentation of the *A.terreus* strain [6]. Alkali nitrate compounds such as potassium and sodium nitrate are not preferred options in the generation of itaconic acid because they promote cell growth and provide high cell biomass instead of acid formation [41], [42]. Moreover, ammonium salt not only promotes acid formation but also decreases the pH value [43] which is beneficial for itaconic acid production. In contrast, an organic compound like yeast extract is not good for acid accumulation as it is utilized in cell growth and promotes high cell concentration and not itaconic acid production. Besides, the concentration of nitrogen should be considered. A high concentration of nitrogen source promotes cell growth and high cell biomass, whereas no nitrogen source in the culture medium leads to cell death because the cell cannot live without nitrogen. Subsequently, the concentration of nitrogen should be kept low to prevent cell growth [7]. The effect of nitrogen was further studied. It was found that when 2.5g/L

of ammonium nitrate was used as the nitrogen substrate, 25g/L of itaconic acid was obtained and 100 g/L of glucose consumed for itaconic acid formation. No itaconic acid was found when the nitrogen presence was too much in the medium culture but a high biomass was released [41].

A phosphate limitation is known for itaconic acid production from fungal fermentation [15]. It decreases the ATP level and the energy charge of the cell leading to the high metabolic flux of glucose via glycosis resulting in itaconic acid formation. Oxidative respiration is uncoupled and is beneficial for itaconic acid production. However, the phosphate level should be kept low to prevent cell growth. Additions of increased concentration of phosphate lead to the mycelial growth (dry weight) and reduce acid production [5].

2.5.2 pH value

The pH value is one factor that should be controlled. Itaconic acid is found in an acid medium and both cis-aconitate and citrate are not present in this environment [28]. Later, there is muchsuccessful itaconic acid production under low pH conditions. It can be concluded that the optimum pH condition of itaconic acid is

a low pH value. For the *A.terreus* strain, rigid control of the pH in the medium at low limits (around 1.8-2.2) is essential for successful itaconic acid formation [29, 41] [4]. During fermentation, the pH value decreases to about 2, and itaconic acid becomes the main product [16]. Moreover, the lower pH value also prevents by-product formation [8] . However, a pH value too low may increase the by-product of itatartaric acid [44]. The pH value also affects cell growth and acid accumulation. At a lower initial pH value, mycelial growth is reduced and is completely inhibited below pH3, whereas above 3.5 the mycelial dry weight tends to increase and acid formation tends to decrease. Itaconic acid was reported found in media after 3 days when the pH value dropped from 3.5 to 1.85 [5]. Normally, on a large scale, such as in a fermentor, the pH is controlled automatically via a pH probe or electrode. However, if the pH value cannot be controlled, like flask culture, itaconic acid formation deepens on the initial pH [13]. The pH value also affects the enzyme of the itaconic acid pathway. An acid environment is preferred for the synthesis of an essential enzyme operating in the conversion of glucose to itaconic acid by *A.terreus* [4]. In contrast, a higher pH value is preferred for cell growth [10]. Another advantage of a

lower pH condition is the prevention of contamination by auto-sterile conditions in the acid environment.

2.5.3 Temperature

The general range of the temperature for filamentous fungal fermentation is between 30° and 37°C for acid biosynthesis fermentation by the *A.terreus* strain with 30°C the optimum temperature. This includes itaconic acid fermentation, optimum itaconic acid production, maximum acidity and mycelial dry weight obtained at 30°C [5].

2.5.4 Aeration and agitation system

Almost all production of itaconic acid on an industrial scale is carried out in a stirred tank reactor (STR). STR is one reactor which can control mixing by the agitation system, dissolved oxygen by the aeration system, temperature, and pH value. STR is preferred for acid fermentation because it provides a good mix of three phases in the fermenter, i.e., the liquid, solid and gas phases. Therefore, there is a good mass transfer in the bioreactor.

Itaconic acid fermentation is an aerobic process. Oxygen is a required condition for itaconic acid production. Oxygen is required for activities of the cell like cell growth and metabolite formation. For the fermentation process, oxygen is transferred into the liquid phase (broth) and the cell can utilize oxygen from only the liquid phase. It is called dissolved oxygen (DO). For itaconic acid formation, DO should be controlled and maintained during all fermentation times. The effect of stopping aeration during the fermentation of itaconic acid was studied. It is reported that when aeration was stopped for 1 minute, there was no effect on itaconic acid formation. However stopping for 3 minutes and there was a 70% fall in the acid production rate compared to the controls, which allowed the aeration system at all times during the fermentation. Stopping for 5 minutes and there was no itaconic formation. However, in the long term, stopping aeration for 3-5 minutes had no effect on itaconic acid formation because after 24 hours acid formation started again [30]. In oxygen-free conditions, cell respiration is blocked leading to the lost ability of growth and metabolite formation. The optimum aeration for itaconic acid may

depend on the strain and culture conditions. On the pilot plant scale, fermentation increases when aeration is controlled at 0.25 vvm. [6].

However, if the aeration rate is too great, then this causes a decrease in the dissolved CO_2. When the dissolved CO_2 is low, there are some effects on itaconic acid formation because oxxaloacitate cannot form under this condition. This leads to low citric acid formation, intermediat itaconic acid formation and, finally, may affect itaconic acid production [45]. In addition, excess aeration also causes foaming. Antifoam is used to solve this problem, but it is not beneficial for itaconic acid. DO also affects cell growth and acid accumulation because DO that is too high might promote cell growth instead of itaconic acid formation.

In order to increase the mixing in the fermentation process, an agitation system is needed. There are three phases in the fermenter: the liquid phase, gaseous phase and solid phase are mixed. Homogenous conditions are important for the fermentation process. Moreover, the agitation system also enhances the transfer between the gas phase and the liquid phase. Oxygen can transfer into the liquid phase and the cell can uptake oxygen from the liquid phase. The agitation system is

beneficial for the fermentation process. Firstly, it prolongs the retention of air bubbles in suspension by reducing bubble size to increase the surface area for oxygen transfer. Next, it prevents bubble coalescence. Additionally, the thickness of the film decreases as the gas-liquid interferes. However, a higher agitation speed leads to shear stress. Conversely, a low agitation rate might exhibit cell flocculation or unwanted growth on surfaces such as on the vessel walls, stirrer and electrode.

Despite the fact that STR is preferred for fermentation, in the case of itaconic acid production by filamentous fungi, some problems do occur, especially, when morphology controlled. This is a major problem for filamentous fermentation. *A.terreus* is a commercial strain for itaconic acid production. Pellet morphology is preferred. Pellets are formed during fermentation. Normally, on a small scale like flask culture it is found that small pellets lead to a high production rate and high concentration of itaconic acid. On a larger scale, small pellet morphology is hardly evident. Large pellets are found in fermenters. These lead to nutrient limitations because the center of the region of a bug pellet may not be surrounded with a medium culture and therefore the cell might lose the ability to grow and produce

metabolites such as itaconic acid. The next problem for itaconic acid production by STR is shear stress. The mixing in the fermenter is controlled by the agitation system. Even high agitation speed can provide good mixing or good oxygen transfer because gas bubbles break down into small bubbles leading to increased oxygen transfer rate (OTR). However, this is the cause of shear stress. For fungal filamentous fermentation, the mycelia are damaged by shear stress bring about cell death and decreased ability for itaconic acid formation. Additionally, viscosity is also a problem for the fermentation process. Oxygen transfer will decrease due to the increase of the viscosity in the culture. Increasing agitation speed might solve this problem [46].

To avoid cell damage from shear stress and mass transfer limitation, an airlift reactor was also tested for itaconic acid production. An ALR can provide good mixing with little shear stress. In 1997, Yahiro et al. reported that ALR could provide a higher production rate at less power input per unit volume than that of STR. In 2L STR, there was a 0.48 g/L/h production rate obtained at 400 rpm of agitation speed and 0.05 vvm. The power input per unit volume for STR was 1180 W/m^3, whereas ALR can reach 0.64 g/L/h of the itaconic acid production rate with the power input per

unit volume at 542 W/m^3. When oxygen enriched air replaces the air for the ALR, the power input per unit volume decreases to 34 W/m^3.

2.5.5 Immobilization technique

All of the problems of itaconic acid fermentation including morphology control and shear stress from STR have been solved. Many researchers have discussed and analyzed the matters at hand. Finally, the immobilization method was tried for itaconic acid formation. The advantage of the immobilization technique is to provide high cell density, as well as to enhance process stability and potential to reuse the immobilized cell. To control morphology cells were immobilized with the solid supporter during fermentation. Alginate bead, polyurethane foam and other supporters were all tested.

In 1985, Kautola et al. investigated the immobilization technique for itaconic acid production by *A.terreus* in a repeat batch. 0/12 g/L/h of productivity was obtained with agar gel. When calcium alginate was used as the supporter, the itaconic acid production rate was 0.06 g/L/h, whereas celite R-626 provided an itaconic acid production rate of 1.2 g/L/h and 11.5 g/L for itaconic acid. The result

shows that the highest production rate was obtained with celite R-626. However, when itaconic acid production is compared between free cell and immobilized cell, it was found that 30 g/L of itaconic acid concentration was achieved and itaconic acid productivity was 0.32 g/L/h. [47]. Polyacrylamide gel was also tested. For this gel it was found that 21 g/L of itaconic acid was released within 10 days. The itaconic acid productivity was 2.8 mg/L/h. [48] ,[49]. In 1986, Ju and Wang addressed the continuous process for *A.terreus* NRRL1960 immobilized in a porous disk bioreactor. It was found that the itaconic acid production rate was 0.73 g/L/h and 18.2 g/L of itaconic acid was released at pH3 [50].

However, itaconic acid production by cells being immobilized cannot compete with acid production by free cells. This is because of mass transfer limitations. The inside of the cell immobilized is not surrounded by a medium and oxygen. In addition, the performance of the immobilized cell depends on the nature of the cell, medium composition and process parameters.

CHAPTER 3

MATERAILS AND METHODS

3.1 Materials

3.1.1 Equipment

Equipment	Model	Company
Autoclave	KT-30 SD	ALP Co., Ltd. (Japan)
5 L stirred tank bioreactor	Biostat B plus	Sartorius
Electronic balance	ML204/01	Mettler Toledo (Switzerland)
Electronic balance	ML 3002E/01	Mettler Toledo (Switzerland)
High Performance Liquid Chromatography (HPLC)	SID 10A	Shimadzu (Japan)
Haemacytometer	-	Bocco Co., Ltd. (Germany)

Laminar flow chamber	NK system clean bench	International Scientific supply (Thailand)
Microscope	Alphaphot-2	Nikon Co., Ltd. (Japan)

Equipment	**Model**	**Company**
pH meter	AB15	Fisher Scientific Pte Ltd. (Singapore)
Rotary incubator shaker	Infors HT Bottmingen	Infors HT (Switzerland)
Ultrasonic sonicator	UD-201	Tomy (Japan)
Vortex mixer	Vortex-genie No. 2	Scientific Industries Inc. (USA)
Hot air oven	UL-80	Memmert Co., Ltd. (Germany)

3.1.2Chemicals

Chemicals	**Company**
Ammonium nitrate (NH_4NO_3)	Merck (Germany)
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck (Germany)
Antifoam	Sigma Aldrich (Germany)
Calcium sulfate ($CaSO_4$)	Sigma Aldrich (Germany)
Corn steep liqour	Sigma Aldrich (Germany)
Copper sulfate ($CuSO_4 \cdot 5H_2O$)	Fluka (Switzerland)
Czapek-Dox agar	Fluka (Switzerland)
Czapek-Dox broth	Fluka (Switzerland)
Glucose ($C_6H_{12}O_6$)	Siamchai Chemical (Thailand)
Itaconic acid ($C_5H_6O_4$)	Sigma Aldrich (Germany)
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Merck (Germany)

Chemicals	Company
Potassium phosphate (KH_2PO_4)	Merck (Germany)
Sodium hydroxide (NaOH)	Merck (Germany)
Sulfuric acid (H_2SO_4)	Merck (Germany)
Yeast extract	Bio Springer (France)

3.2 Microorganism and medium compositions

A.terreus NRRL1960 used in this study was kindly obtained from the Agricultural Research Service culture collection, US Department of Agriculture, Peoria, IL, USA. The stock culture was kept on the Czapek-Dox agar plate. For inoculum preparation, the stock culture was transferred onto the freshly new agar plate and then incubated at 30°C for 7 days. The spores were collected from the 7^{th} day agar plate and suspended into the sterile DI water. The spore concentration was determined using a hemacytometer (Appendix A). The spore suspension at 10^7 spores/mL was then prepared by diluting with the sterile DI water.

In this study, itaconic acid production consisted of 2 phases, i.e. growth and production phases. Different medium recipes were tested for suitable growth and acid production. All recipes were listed below.

Growth medium (GM)

GM1 [11]

Glucose	55	g/L
NH_4NO_3	5	g/L
CSL	3	g/L
$Mg_2SO_4 \cdot 7H_2O$	2	g/L

GM2 [10]

Glucose	20	g/L
$(NH_4)_2SO_4$	0.47	g/L
KH_2PO_4	0.022	g/L
$MgSO_4 \cdot 7H_2O$	0.415	g/L

GM3		
Glucose	30	g/L
Yeast extract	5	g/L
GM4 [50]		
Glucose	50	g/L
$(NH_4)_2SO_4$	3.3	g/L
$MgSO_4 \cdot 7H_2O$	0.8	g/L
KH_2PO_4	0.044	g/L
$CuSO_4 \cdot 5H_2O$	0.004	g/L
GM5 [6]		
Glucose	66	g/L
$(NH_4)_2SO_4$	2.7	g/L
$MgSO_4 \cdot 7H_2O$	0.8	g/L

Production medium (PM)

PM1 [6]

Glucose	66	g/L
$(NH_4)_2SO_4$	2.7	g/L
$MgSO_4 \cdot 7H_2O$	0.8	g/L
CSL	1.8	g/L

PM2 [50]

Glucose	50	g/L
$(NH_4)_2SO_4$	3.3	g/L
$MgSO_4 \cdot 7H_2O$	0.8	g/L
KH_2PO_4	0.088	g/L
$CuSO_4 \cdot 5H_2O$	0.004	g/L

PM3 [9]

Glucose	100	g/L
$(NH_4)_2SO_4$	3.0	g/L

$MgSO_4 \cdot 7H_2O$	0.5	g/L
$CaSO_4$	3	g/L
PM4 [10]		
Glucose	60	g/L
$(NH_4)_2SO_4$	2.36	g/L
KH_2PO_4	0.11	g/L
$MgSO_4 \cdot 7H_2O$	2.1	g/L

3.3 Batch fermentation study in the shaken flask

As previously mentioned, itaconic acid production by *A. terreus* NRRL1960 consisted of growth and production phase. The growth phase was started by inoculating 0.5 mL spore suspension (10^7 spores/mL) into 50 mL sterile growth medium. The culture was incubated at 30°C for 3 days. At the end of the growth phase, the growth medium was discarded and replaced with 50 mL freshly sterile production medium. Then the culture was further incubated for 7 days. During fermentation, the samples were collected every 12 h for further analyses of the remaining glucose and end products.

In this section, the effects of medium compositions (both growth and production media), the initial culture pH, the rotational speed on the fermentation kinetics were observed. In addition, free cells and immobilized cells (on a 5×5 cm^2 cotton cloth) were compared for the ability to grow and ferment glucose to itaconic acid.

3.4 Free cell fermentation in the 5 L stirred bioreactor

Similarly to 3.3, the fermentation optimization was performed in the bioreactor scale. The media optimized in 3.3 were used in the fermentation study in the bioreactor. The growth medium consisted of 30 g/L glucose and 5 g/L yeast extract while the production medium contained 66 g/L glucose, 2.7 g/L $(NH_4)_2SO_4$, 0.8 g/L $MgSO_4 \cdot 7H_2O$.

Before fermentation started, the bioreactor containing 3 L growth medium was autoclaved at 121°C, 15 psig for 45 min. After autoclave and cool down, the temperature was set up at 30 °C and the dissolved oxygen probe was calibrated with nitrogen and air. The initial pH was adjusted to 3. Later, 10 mL of 10^7 spore/mL spore suspension were transferred into the bioreactor. Spore suspensions were

allowed to germinate in growth medium for 3 days. After that the growth medium was discarded and the bioreactor was filled up with 3 L sterile production medium. During the production phase, the pH was automatically controlled at 2. NaOH and H_2SO_4 were used for pH control. The production phase took 7 days. The agitation and aeration rates were varied. Samples were taken every 12 h for further analyses.

3.5 Immobilized cell fermentation in the 5 L stirred bioreactor

Fermentation using immobilized cells was carried out in the bioreactor. A pre-weighed cotton cloth was affixed with the baffle in the 5 L stirred bioreactor where the fungal spores germinated and immobilized. The bioreactor was prepared and set up similarly to that mentioned in 3.4. 10 mL of 10^7 spore/mL spore suspension were transferred into the bioreactor containing 3 L sterile growth medium. Spore suspensions were allowed to germinate and immobilize on the cotton cloth during the growth phase for 3 days. Later, the growth medium was discarded and the bioreactor was filled up with 3 L sterile production medium. During the production phase, the pH was automatically controlled at 2. NaOH and H_2SO_4 were used for pH

control. The production phase took 7 days. The agitation and aeration rates were varied. Samples were taken every 12 h for further analyses.

Cell immobilization in the calcium alginate was also studied. The immobilized beads were prepared by dissolving the spore suspension (10^8 spores/mL) into calcium alginate solution (4% w/v). The volume ratio of spore suspension to calcium alginate solution was 1:3. The mixture was stirred thoroughly to ensure rigorous mixing. The gel beads were prepared by dropping 10 mL of the mixture into 0.15 M $CaCl_2$ solution followed by rigorous stirring for 30 min. The beads were filtered and washed with sterile DI. Later, the beads were transferred into the bioreactor. The spores entrapped in the gel beads were allowed to germinate and grow in the growth medium for 3 days. Later, the growth medium was discarded and the bioreactor was filled up with 3 L sterile production medium. During the production phase, the pH was automatically controlled at 2. NaOH and H_2SO_4 were used for pH control. The production phase took 7 days. The agitation and aeration rates were varied. Samples were taken every 12 h for further analyses.

3.6 Analytical methods

3.6.1 Cell dry weight

The culture broth was filtered through filter paper (whatman no.1) to separate the mycelia and other suspended solids from the supernatant. The cell on the filter paper was washed thoroughly with DI water and then 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:

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

3.6.2 Remaining glucose and end products

The supernatant was analyzed for the residual glucose, itaconic acid, and other byproducts using high performance liquid chromatography (HPLC). Prior to HPLC analysis, the supernatant was diluted with DDI water and filtrated through the cellulose acetate membrane (0.2 μm). 15 μL diluted particle free sample was automatically injected into an organic acid analysis column (Biorad Aminex HPX-87H ion exclusion organic acid column: 300 mm × 7.8 mm) maintained at 50°C in a column oven (Shimadzu; CTO-10A). 0.004 M H_2SO_4 was used as an eluant at 0.4 mL/min flow rate (Shimadzu; LC-10Ai). A refractive index detector (Shimadzu; RID-

10A) was used to detect the organic compounds. The standards containing glucose, itaconic acid, citric acid, and cis-aconitate at various concentrations from 0.25 g/L to 2.00 g/L were injected as the references for determining the concentration of each component in the sample. The peak area was used for the comparison basis.

3.7 Determination of volumetric oxygen transfer coefficient

3.7.1 Determination of K_La in flask culture using sulfite oxidation method

50 mL of 5% Na_2SO_3 and 0.32 g/L of $CuSO_4$ were added into 250 mL Erlenmeyer flask. Next, incubated at 30 °C 200 and 250 rpm. The sample was collected every 10 min from the beginning to 60 min. Add dry ice in the sample immediately after the sample were collected. 1 mL of sample was titrated with standardized I2 solution and using 1% of starch solution as an indicator. In order to determine K_La, concentration of Na_2SO_3 and times were plotted.

3.7.2 Determination of K_La in 5L bioreactor using gassing out technique

The 5L of bioreactor containing 3L of production medium and operated at 30 °C 0.05vvm. Agitation rate was controlled at 300 rpm. After the value of DO is stable stop aeration and N_2 were filled. Record the values of DO every 10 second until constant DO is reached. After that, stop filling N_2 and resume aeration rate to

0.5 vvm and record the value of DO every 10 second until constant DO is reached. Change the agitation rate to 500 and 700 rpm for comparison. In order to determine the K_La, using the data during air in period before reaching steady state to calculate ln (C_L*-C_L) and then plot ln (C_L*-C_L) versus time.

CHAPTER 4

RESULT AND DISCUSSION

In this thesis, itaconic acid production by *A.terreus* NRRL1960 was investigated under different fermentation conditions. The key parameters studied were determined and their effects on growth and fermentation kinetics were discussed.

4.1 optimization of medium and pH optimization for promoting growth

Unlike other common microorganisms, it was claimed that *A. terreus* grew and produced itaconic acid at acidic pH to facilitate acid transport throughout the cells [51]. In this section, the effects of medium and pH were studied. Initially, the medium and pH were optimized during the growth in order to achieve high cell biomass content for producing itaconic acid later in the production phase.

In previous study, one phase fermentation for itaconic acid was carried out at low pH. The low acid productivity might have been due to low cell machinery to produce itaconic acid since microorganism generally grows at neutral pH. When growing and producing itaconic acid at the same time at a certain pH, this either

promote cell growth or enhance itaconic acid production. In this work, the 2-phase fermentation approach was attempted. Initially, the medium and the pH were optimized to promote growth. There were 5 media tested with three different initial pH for the improved growth. The medium compositions were selected from previous study in the topic of itaconic acid production by *A. terreus*. In growth phase, *A. terreus* NRRL1960 were cultured in 250 mL Erlenmeyer flask containing 50 mL growth medium. The temperature was controlled at 30°C and shaken at 150 rpm. Tables 4.1-4.5 show glucose consumption and cell growth obtained from different media.

From the results obtained, GM3 gave the highest cell biomass production regardless to pH. It was also found that the highest glucose consumption was observed from the culture in this medium. The only difference in GM3 compared to other growth media was yeast extract as the sole nitrogen source. Unlike other itaconic acid production process that was commonly one phase fermentation. In this work, 2 phase approach was introduced. Yeast extract was commonly used for initiating and enhancing growth. The results obtained herein confirmed the hypothesis of 2 phase fermentation as the highest cell production could be achieved

during the growth phase when cultivating *A. terreus* in GM3. GM3 contained yeast extract whereas the inorganic ammonium nitrogen was present in other media. Yeast extract contains not only nitrogen but also other ingredients necessary for growth including essential amino acids and vitamins; thus, helped promote cell biomass production by *A. terreus*. While inorganic nitrogen source was preferred for acid formation [52]. Although it seemed that the change in the initial pH did not strongly affect cell biomass production, previous study done by Riscaldati et al. (2000) claimed that at the pH of 3.0 compared to other pH studied facilitated oxygen transport; thus, leading to high biomass production. Therefore, in further study, pH 3.0 was selected. Moreover, the low of pH value is required for itaconic acid production. So, that's mean there's a little difference of pH value between growth and itaconic acid production phase.

Table 4.1 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM1 for 3 days.

pH	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	$Y_{x/s}$ (g/g)
3	53.61	26.84	6.74	0.25
4	62.22	15.92	4.69	0.29
5	55.51	13.89	4.87	0.35

Table 4.2 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM2 for 3 days.

pH	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	Yx/s (g/g)
3	18.06	7.48	0.86	0.11
4	17.63	10.06	1.05	0.10
5	23.61	3.91	1.17	0.30

Table 4.3 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM3 for 3 days.

pH	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	Yx/s (g/g)
3	33.87	29.21	7.42	0.25
4	31.19	25.56	6.72	0.26
5	32.57	22.99	7.27	0.32

Table 4.4 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM4 for 3 days.

pH	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	Yx/s (g/g)
3	46.15	7.48	1.67	0.22
4	48.55	10.06	1.64	0.16
5	45.39	3.91	1.67	0.43

Table 4.5 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM5 for 3 days.

pH	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	Yx/s (g/g)
3	62.35	17.89	5.12	0.29
4	59.24	8.81	3.96	0.45
5	58.76	18.44	4.22	0.23

4.2 Effect of rotational speed on cell growth in growth phase

From last experiment, it was proven that GM3 was selected for initiating growth at pH 3. In this experiment, further optimization on proper rotational speed was determined (Table 4.6). It was found that the highest cell biomass was obtained when the rotational speed was controlled at 250 rpm. Nonetheless the 4 rotational speeds studied did not show much difference in glucose consumption for cell

biomass as observed from the final cell concentration and the biomass yield. However, to ensure sufficient oxygen supply for growth the highest speed was selected for further fermentation study due to the general principle on good mixing and high oxygen transfer that promoted cell growth. Nonetheless, it should be aware of excess biomass concentration which eventually led to highly viscous broth and change in hydrodynamic behavior (from Newtonian to non Newtonian) [53].

In summary, for sufficiently high amount of cell biomass passing towards itaconic acid production phase, *A. terreus* was grown in GM3 at pH 3 and 250 rpm for 3 days. This operating condition was further used in the next experiment to determine the proper condition to promote itaconic production [5].

Table 4.6 Glucose consumption and biomass production obtained during the cultivation of *A. terreus* in GM3 at pH 3 with various rotational speeds for 3 days.

Speed (rpm)	Initial glucose (g/L)	Glucose Consumption (g/L)	Cell concentration (g/L)	$Y_{x/s}$ (g/g)
100	30.00	17.72 ± 0.15	5.12 ± 0.03	0.29 ± 0.00
150	30.00	29.21 ± 0.65	7.42 ± 0.11	0.25 ± 0.00
200	30.00	25.96 ± 1.13	7.20 ± 0.18	0.27 ± 0.11
250	30.00	28.05 ± 0.57	7.58 ± 0.29	0.27 ± 0.01

4.3 Determining the proper medium to enhance itaconic acid production

At the end of the growth phase, the growth medium was discarded and the culture was filled up with the sterile production medium. During the production phase, the optimized pH was initially set at 2 as reported in the previous literatures. The culture was incubated for 7 days at 30 °C. Table 4.7 shows the fermentation kinetics of *A. terreus* cultivated for itaconic acid production using different media. Among other media studied, PM1 contained CSL an organic nitrogen source. It was

presumably that the growth factor contained in CSL helped promote glucose flux towards TCA cycle; thus, some amount could be released for itaconic acid production. This supported the finding that cultivating *A. terreus* in PM1 gave the highest itaconic acid production. Compared with the theoretical yield (0.72 g itaconate per g glucose), the yield obtained from the cultivation using PM1 was approximately 48.9%. Therefore, PM1 was selected for further optimization step.

Table 4.7 Effect of medium compositions on itaconic acid production by *A. terreus* cultivated at 30 °C, pH 2, and 200 rpm for 7 days

Medium	Glucose consumption (g/L)	Cell concentration (g/L)	Yx/s	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
PM1	61.85±0.06	12.47±0.13	0.20 ± 0.00	21.98±0.89	0.35±0.01	0.13±0.01
PM2	46.52±0.02	12.29±0.71	0.26±0.01	13.25±0.76	0.28±0.01	0.07±0.00
PM3	89.21±0.23	17.45±0.41	0.19±0.04	12.65±0.62	0.14±0.07	0.08±0.00
PM4	58.68±0.86	13.39±0.82	0.23±0.02	17.22±0.31	0.29±0.00	0.10±0.00

In this study, the initial pH was set at 2.0 because *A. terreus* produced itaconic acid as the main product at this pH [16]. At this low pH (lower than pH 3.0), it helped suppress the formation of the byproducts [8]; thus, the metabolic flux redistributed toward itaconic acid production. The higher pH value above 3.0 allowed better oxygen transfer that promoted high cell biomass production instead of itaconic acid. While at the low initial pH, the cell growth was limited and rather inhibited at the pH lower than 3.0 [5]. In flask culture, the pH value was hardly controlled so that itaconic acid formation depended on the initial pH value [13] ,[54].

4.4 Effect of rotational speed on itaconic acid production

From previous results, it was found that higher rotational speed could promote growth; however, in itaconic acid synthesis, completing TCA cycle lowered itaconic acid production as the bypassed flux of citrate isomer towards itaconic acid synthesis was limited. Therefore, high rotational speed was still necessary at certain level in order that pyruvate flux could enter TCA cycle at the early stage and later it was bypassed for itaconic acid [45]. Table 4.8 indicated the effect of rotational speed on itaconic acid production. It was clear that increasing rotational speed to 250 rpm

caused adverse effect on itaconic acid production while cell biomass production was promoted during itaconic acid production phase. That's because at 250 rpm of rotation speed is provide too much oxygen. It was utilized for cell growth not itaconic acid formation. Excess of oxygen is not required for itaconic acid production.

Table 4.8 Effect of rotational speed on itaconic acid production by *A. terreus* cultivated in PM1 at 30 ℃ and pH 2 for 7 days

Speed (rpm)	Glucose consumption (g/L)	Cell concentration (g/L)	Yx/s	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
200	61.85±0.06	12.47±0.13	0.20±0.00	21.98±0.89	0.35±0.01	0.13±0.01
250	53.02±0.58	17.38±0.05	0.33±0.00	10.38±0.28	0.20±0.03	0.06±0.00

In summary, the well-suited conditions that promoted itaconic acid production in the flask culture of *A. terreus* were at pH 2, 200 rpm in PM1 medium.

Figure 4. 1 Morphology of *A.terreus* in PM1, pH2, 200rpm and 30 ℃ at 7th day production phase. Before production phase, *A.terreus* was cultured in GM3 at pH3 for 3 days then growth medium was removed and replaced with production medium; PM1.

4.5 Immobilization of *A. terreus* on a cotton cloth

In fungal fermentation, immobilization was proven to be the effective way to control proper morphology; thus, enhance the production rate. *A. terreus* spores were inoculated in GM3 with the presence of cotton cloth where the spores were expected to immobilize and germinate. The growth phase took 3 days. Later, the growth medium was replaced by the production medium and the culture was incubated at the same conditions reported earlier. Table 4.9 shows the fermentation kinetics of immobilized cells compared with that of the free cells. From the results it

was found that immobilization on the cotton cloth did not promote itaconic acid production. This is presumably due to improper morphological control on the cotton cloth.

Figure 4. 2 Morphology of immobilized *A.terreus* on cotton cloth in PM1, pH2, 200 rpm and 30 ℃ at 7th day production phase. Before production phase, *A.terreus* was cultured in GM3, 30 ℃ at pH3 for 3 days then growth medium was removed and replaced with production medium; PM1.

Table 4.9 Comparing the efficiency of immobilized cells on a cotton cloth with free cells cultivated in a shake flask

	Glucose consumption (g/L)	Cell concentration (g/L)	Yx/s	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
Free cells	61.85±0.06	12.47±0.13	0.20±0.00	21.98±0.89	0.35±0.01	0.13±0.01
Imb cells	39.80±0.46	13.53±0.22	0.34±0.00	8.14±0.20	0.20±0.02	0.05±0.01

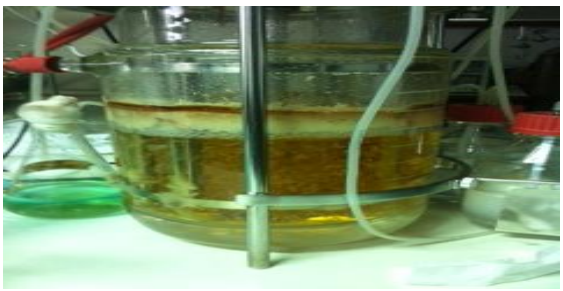
Owing to the limited oxygen transfer as well as substrate and product transport in the immobilized cells for itaconic acid, lower itaconic acid production was obtained in the immobilized cell culture. This was because the transport throughout the dense sheet of fungal cells immobilized on the cotton cloth was hindered compared with the loose and small pellets formed in free cell cultivation [52].

4.6 Itaconic acid production in a 5 L stirred bioreactor

To further increase itaconic acid production, fermentation in a 5 L stirred bioreactor was conducted as it could be able to control the key operating factor pH precisely in the bioreactor operation as compared to that in the flask culture. As previously study, it was found that rotational speed was another key factor relating to the oxygen supply for TCA and the bypass of TCA intermediate towards itaconic acid. Table 4.10 shows the fermentation kinetics of *A. terreus* cultivated in the stirred bioreactor using GM3 for growth and PM1 for inducing itaconic acid production. The pH was controlled at 3 during the growth phase while it was lowered to 2 during the production phase. The fermentor was aerated at 0.5 vvm. It was found that at 300 rpm, no itaconic acid was produced while glucose was consumed. Increasing the agitation speed led to an increase in itaconic acid; however, the amount was very low (Table 4.10).

It is clear that growing *A. terreus* in the 5 L stirred bioreactor for itaconic acid was not as good as that in the shake flask. Basically, stirred bioreactor provides better control in term of temperature, pH, dissolved oxygen and so on. Although stirred

bioreactor could provide good mixing and oxygen transfer, itaconic acid fermentation by *A. terreus* somewhat experienced the difficulty in operation. Improper morphological control lowered oxygen transfer due to highly viscous broth [46]. Increasing agitation speed commonly improves oxygen transfer; however, high power input enhanced hydrodynamic stress which eventually leads to mycelial damage and loss of ability to grow and produce itaconic acid [19]. On the other hand, low agitation speed might generate cell flocculation. It was found in this work that cell grew at the surfaces of stirrer, probes, and walls. Only a small amount of cell immerged in the liquid broth. This might be the cause of the difference in cell behavior compared to that in the shake flask culture resulting in low itaconic acid production.



(a)

(b)

(c)

Figure 4. 3 Morphology of free cell *A.terreus* in the medium PM1, pH2 and 30 °C (a) with 300 rpm of agitation speed at 7^{th} days production phase; (b) with 500 rpm of agitation speed at 7^{th} days production phase; and (c) with 700 rpm of agitation speed at 7^{th} days production phase.

Table 4.10 Itaconic acid production by free cells of *A. terreus* in the stirred fermentor . Before entering production phase, *A.terreus* was grown in GM3 at 30 ℃, pH 3 for 3 days then GM1 was removed and replaced with PM1 for production phase. During the production phase controlled at 30 ℃ and pH2 in medium PM1 for 7 days.

Speed (rpm)	Glucose consumption (g/L)	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
300	18.91±0.06	0	0	0
500	26.81±0.21	1.03±0.02	0.04±0.00	0
700	11.88±0.73	1.13±0.07	0.09±0.00	0

4.7 Immobilizing *A. terreus* on the cotton cloth affixed on the static bed in the 5 L fermentor

After inoculation with the spores, *A. terreus* germinated and immobilized onto the cotton cloth affixed on the static bed. This resulted in the clear fermentation broth. It was presumed that with better morphological control by way of immobilization for the bioreactor operation should have improved the fermentation

performance compared to those obtained from the free cells cultivated in the stirred fermentor. However, the results shown in Table 4.11 indicated improper morphology of *A. terreus* for itaconic acid production in the fermentor scale. From Table 4.11, it is clear that increasing agitation resulted in more glucose consumed but those were not for itaconic acid. Glucose consumption might be for cell biomass and ATP regeneration so that *A. terreus* could survive under the improper morphology [52]

Figure 4. 4 Morphology of *A.terreus* immobilized on the cotton cloth in the static bed bioreactor at 30 ℃ and pH2 for 7 days.

Table 4. 11 Itaconic acid production by *A. terreus* immobilized on the cotton cloth in the static bed bioreactor. Before entering production phase, immobilized *A.terreus* was grown in GM3 at 30 °C, pH 3 for 3 days then GM1 was removed and replaced with PM1 for production phase. During the production phase controlled at 30 °C and pH2 in medium PM1 for 7 days.

Speed (rpm)	Glucose consumption (g/L)	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
300	6.12 ± 2.03	1.20 ± 0.01	0.22 ± 0.07	0
500	17.79 ± 0.66	1.01 ± 0.01	0.06 ± 0.00	0.01 ± 0.00
700	38.98 ± 1.23	0.88 ± 0.04	0.02 ± 0.00	0.01 ± 0.00

The results obtained in this part of the study revealed that unlike what was hypothesized that immobilization in the form of thin fungal sheet on the cotton cloth would not only provide the better morphological control that eased the operation in term of medium change and product recovery but also improve itaconic

acid production. However, it was reported that higher itaconic acid production was obtained from the small pellets [30]. In 4.3, it is clear that the self forming pellets gave the high itaconic acid production. From the literatures and findings in this study, it was believed that pellet was the preferred form of *A. terreus* for itaconic acid production. Immobilization in the form of fungal sheet might result in mass transfer limitation. During fermentation, cells continuously grew on the cotton cloth from monolayer to multilayer covered on the cotton cloth. This eventually led to the difficulty to control the thickness of the fungal sheet [55]. The thickness of fungal cells immobilized on the cotton cloth hindered both the transfer of oxygen and essential substrates and the product to be transported into the broth.

4.8 Using the immobilized *A. terreus* pellets to produce itaconic acid in the stirred fermentor

In this part, immobilized *A. terreus* was prepared by entrapping the spores in calcium alginate beads for itaconic acid production as of the previous finding and literatures claimed the pellets were the preferable form of *A. terreus* for itaconic acid synthesis. Table 4.12 shows the production kinetics of pelletized *A. terreus* in the

stirred fermentor. Compared to those reported in the fermentor level experiments, immobilized *A. terreus* in calcium alginate beads provided a higher itaconic acid production though the values obtained were still low compared to the results obtained in the flask scale in this study. From this, it could be concluded that pellet was the preferred morphology for itaconic acid synthesis by *A. terreus*. Unlike fungal adsorption on the cotton cloth surface, entrapment in the gel bead matrices gave the well suited transport of substrates and products throughout the immobilized structure. Nonetheless, the conditions provided in the fermentor were somehow different from those in the flask culture that resulted in the lower itaconic acid production.

(a)

(b)

Figure 4. 5 Morphology of the pelletized *A.terreus* in calcium alginate beads in medium PM1, 30 °C and pH2 (a) with 100 rpm of agitation speed at 7th day production phase and (b) with 300 rpm of agitation speed at 7th days production phase.

Table 4.12 Itaconic acid production by the pelletized *A. terreus* in calcium alginate beads in the stirred bioreactor Before entering production phase, the pelletized *A.terreus* in calcium alginate beads was grown in GM3 at 30 ℃, pH 3 for 3 days then GM1 was removed and replaced with PM1 for production phase. During the production phase controlled at 30 ℃ and pH2 in medium PM1 for 7 days.

Speed (rpm)	Glucose consumption (g/L)	Itaconic acid concentration (g/L)	Yp/s	Productivity (g/L·h)
100	1.19 ± 0.75	3.35 ± 1.18	3.65 ± 1.31	0.02 ± 0.01
300	13.17 ± 1.01	0.83 ± 0.01	0.06 ± 0.01	0

From previous runs in the fermentor scale, it was obvious that high agitation resulted in higher glucose consumption with less itaconic acid accumulated. In this part, lower agitation rate was used and the results confirmed the hypothesis of using high agitation resulted in higher glucose consumption and lower itaconic acid production. Lower agitation rate to 100 rpm gave an improved production of itaconic

acid though the amount was still very low. From Fig. 4.6, when oxygen supply was sufficient, glucose was converted toward glycolysis at the high rate for pyruvate. Pyruvate was then entering tricarboxylic acid cycle coupled with an electron transport chain where oxygen was a final electron acceptor for cell biomass synthesis and ATP regeneration. Therefore, glucose flux was driven toward cell production instead of bypassing for itaconic acid production.

Although pellets were prepared by immobilizing in the gel beads, *A. terreus* pellets in the stirred fermentor were rather different from those self-immobilized in the shake flask culture. Although the low agitation was attempted in the stirred fermentor, the hydrodynamic condition was not similar to that appeared in the shake flask. Oxygen transfer as well as mixing pattern might be responsible for metabolism of *A. terreus*. Excess oxygen though agitation was lower in the fermentor rather drove the glucose flux towards completed TCA for cell biomass and ATP regeneration. Also, it was reported that immobilized cells in the pellet form gave lower itaconic acid production compared to the self forming pellets [47]. Therefore, to improve itaconic acid production, further investigation on mixing pattern to drive glucose flux toward

itaconic acid synthesis as well as morphological control to achieve much similar pellet structure to that in the free cell culture

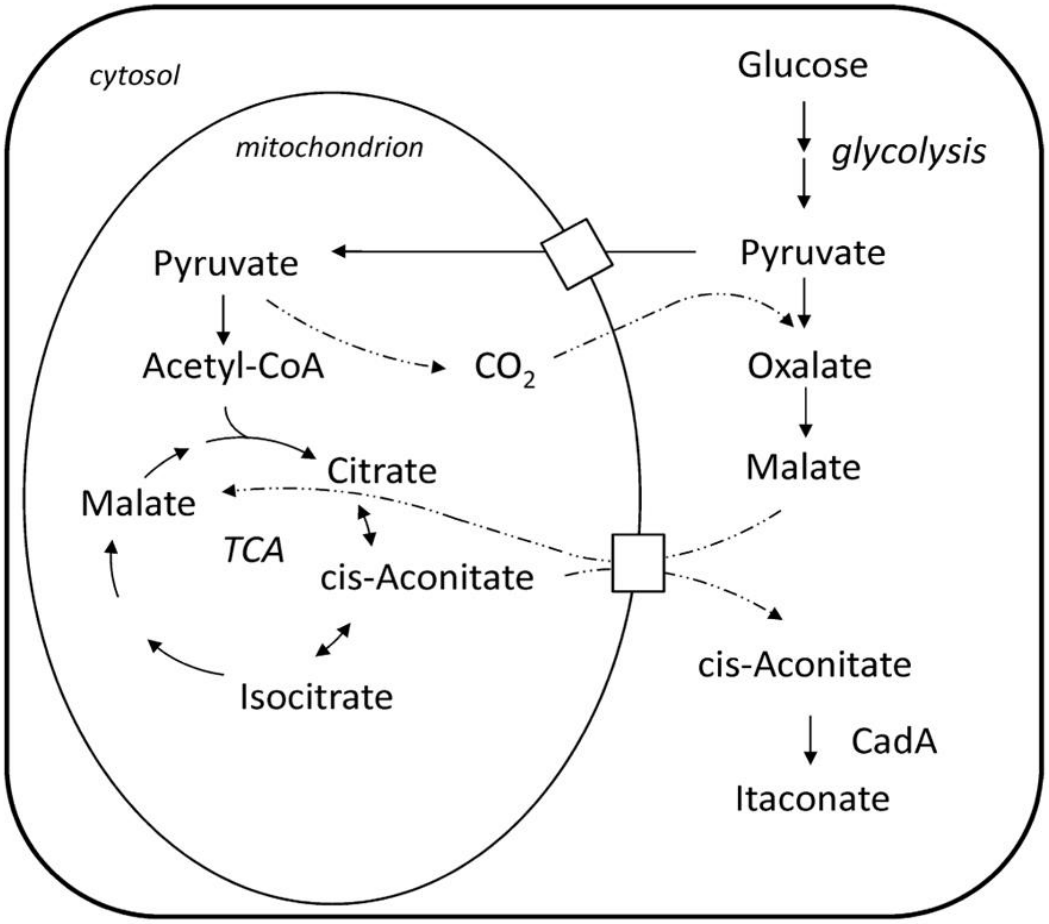


Figure 4.6 Biosynthesis pathway of itaconic acid in *A. terreus*. Malate/Citrate transport during nitrogen limitation played role controlling itaconic acid synthesis. (Reprinted from Steiger et al. (2013). [56]

4.9 Determination of the volumetric oxygen transfer coefficient

From previous results, it was noted that oxygen might be responsible for itaconic acid production. Low itaconic acid production obtained in the bioreactor operation might be due to the improper oxygen tension. In this part, oxygen transfer coefficient (K_La) was measured at the agitation speeds studied in both flask and fermentor scales and compared (Table 4.13).

Table 4. 13 K_La at different speed from the shaken flask and stirred fermentor

Speed (rpm)	K_La (min^{-1})	
	Shake flask[a]	Stirred fermentor[b]
200	1.43±0.29	n/a
250	1.70±0.51	n/a
300	n/a	0.87±0.02
500	n/a	1.52±0.01
700	n/a	2.02±0.22

Remarks:

[a] K_La was measured by sulfite oxidation

[b] K_La was measured by static gassing out

From Table 4.13, it was clear that higher speed yielded higher K_La. Increasing rotational speed from 200 rpm to 250 rpm gave slightly low itaconic acid but more biomass formation. This confirmed the hypothesis of high oxygen driving glucose flux toward completed TCA coupled with ETC instead of bypassing the TCA for itaconic acid production.

Compared to the shake flask culture, K_La values in the stirred fermentor were lower. Though the K_La value in the stirred fermentor operated at 500 rpm was somewhat similar to that in the shake flask cultivated at 200 rpm, itaconic acid was not produced in the stirred fermentor due to the different morphology. Diverse morphology in the stirred fermentor during cultivation might be another cause of lowered itaconic acid synthesis. Thus, it can be summarized that appropriate morphology is necessary for inducing itaconic acid production. In addition, sufficient

but not excess oxygen promoted itaconic acid as the glucose flux was driven toward itaconic acid synthesis.

CHAPTER 5

CONCLUSION AND SUGGESTION

In this study, the optimal medium for *A. terreus* NRRL1960 was elucidated. It was found that the inorganic compound as the nitrogen source in the itaconic acid fermentation was preferable. Under low pH, the higher cell growth as well as itaconic acid was observed. Nonetheless, changing the rotational speed did not affect much on itaconic acid production.

The result shown that medium composition has effect on cell growth in growth phase and itaconic acid production in production phase. When yeast extract were used as nitrogen source, the maximum biomass were presence during growth phase. Yeast extract were preference and easy to use for cell growth. In addition, yeast extract also containing nitrogen, protein, amino acid, vitamins and minerals which necessary and needed for cell growth. However, inorganic compounds are affective not only for cell growth but also for itaconic acid formation in production phase. The maximum itaconic acid concentration were released when corn steep

liquor were used as nitrogen source in production phase. To suppress formation of other organic acid might be appear in TCA cycle, the pH –values of medium should be kept low. Moreover, itaconic acid was produced at low pH-values.

Itaconic acid production is an aerobic process. There's generally agreed that oxygen were needed for itaconic acid production. In flask culture, a higher of rotation speed brings about higher of cell biomass in growth phase not for itaconic acid formation in production phase because oxygen were used for cell growth instead itaconic acid formation. Nevertheless, 5L bioreactor was tested for itaconic acid production by fermentation with *A.terreus* NRRL 1960 but without success. The cells were damaged in stirred tank reactor and decrease ability to produce of itaconic acid

Morphology was also observed. In comparison between flask cultured and 5L bioreactor, the result shown that the flask culture provide a small pellet of *A.terreus* and release high yield of itaconic acid. During 5L bioreactor, small pellets were not appearing and release a low of itaconic acid production. It might be conclude that a small pellet is good morphology to produce itaconic acid. To control morphology of

cells, immobilize technique were tried. The cells were entrapped in alginate bead. Even cell-alginate mixture brings about a small pellet in stirred tank reactor but still without success of itaconic acid production. The concentrations of itaconic acid were still low in experiment with alginate bead.

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APPENDIX

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

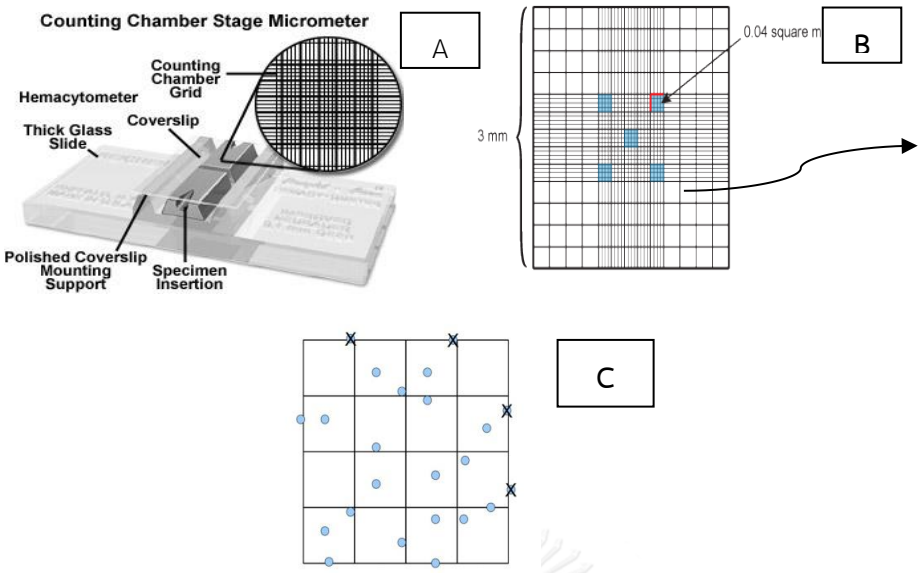

Figure A1 The hemacytometer with coverslip (A); the counting chamber consists of 25 large squares and each large square is divided into 16 small squares, each 0.2 mm on a side, or 0.04 mm^2 (B); count and un-count cells touching the edge lines (C).

VITA

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