รายงานการวิจัย

เรื่อง

การพัฒนาเทคโนโลยีการผลิตกรดอิทาโคนิคเพื่อการผลิตพลาสติกชีวภาพโดยเซลล์ตรึงของ *Aspergillus terreus* บนเส้นใยธรรมชาติในถังปฏิกรณ์ชีวภาพแบบเบดสถิต

Development of itaconic acid production technology for bioplastic using immobilized *Aspergillus terreus* on natural fiber in the static bed bioreactor

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Abstract

In this work 2 phase fermentation approach was firstly introduced in itaconic acid production. Medium optimization indicated that during growth phase organic nitrogen provided the highest cell biomass production while in the production phase, slight amount of organic nitrogen helped drive the pyruvate flux towards TCA; thus, it could be bypassed to itaconic acid production. Morphology also played key role in itaconic acid production. From the results obtained in this study, pellet structure was a preferred structure that promoted itaconic acid production in *A. terreus*.

บทคัดย่อ

ในงานวิจัยนี้ ได้ใช้วิธีการหมักแบบสองขั้นตอนเป็นครั้งแรกในการผลิตกรดอิทาโคนิค จากการทดลองปรับสูตรอาหาร พบว่า ในช่วงการเจริญเติบโต แหล่งไนโตรเจนอินทรีย์ช่วยให้ผลิต ชีวมวลได้ ที่สูงที่สุด ในขณะที่ ขั้นตอนการผลิตไนโตรเจน อินทรีย์ จำนวน เล็กน้อยของ ช่วยผลักดัน การไหลของ ไพรูเวทเข้าสู่วัฏจักรเครบส์ ซึ่งจะมีฟลักซ์บางส่วนไหลเข้าสู่การผลิตกรดอิทาโคนิค นอกจากนี้ ยังพบว่าสัณฐานวิทยา ยังมี บทบาทสำคัญใน การผลิตกรด itaconic จากผล ที่ได้รับในการศึกษาครั้งนี้ โครงสร้างแบบเพลเล็ท เป็น โครงสร้าง ที่สามารถให้ผลผลิตกรดดีที่สุด

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Introduction

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 fungi 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, *Aspergillus 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 is the most plausible one. The main route is via glycolysis pathway and tricarboxylic acid cycle. (Kinoshita, 1931; Bentley and Thiessen, 1957; Winskill, 1983) 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 reactions were found in TCA cycle like citric acid dehydration reaction. Finally, itaconic acid was formed from aconitate decarboxylase. The second pathway that referred is converting 1,2,3-tricarboxypropen to itaconic acid. (Shimi and Nour El Dein, 1962) The last suggested pathway considered the condensation of pyruvate and acetyl Co-enzyme A to citramalate.(Nowakowska – Waszczuk, 1973) 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 factors 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. (Eimhejejjn and Jarsen, 1955; Elnaghy ang Megalla, 1975) Not only the carbon source but also the concentration of carbon affects itaconic acid production. It was found that 5-7%(W/V) of glucose was preference. (Prescott and Dunn, 1959) Nitrogen source also plays role 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. (Pfeifer, 1952) 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 cannot be alive and not found itaconic acid in this case. (Milson and Meers, 1985) 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. (Batti, 1964) Between 1.8-2.1 is preferable for itaconic acid production because it's optimum pH for enzyme activities in itaconic acid pathway. (Lockwood and Nelson, 1946; Larsen and Eimhjellan, 1995; Bentley and Thiessen, 1957) Itaconic acid production was not presence when pH value is higher than 6. Last but not least, the temperature have affected on itaconic acid production. 30

degree Celsius of temperature is preferred for itaconic acid production. The last factor is oxygen, which is required for itaconic acid production and cell growth especially filamentous fungi.

There have been researches regarding the itaconic acid production from *Aspergillus terreus* . For example, In 1945 Lockwood and Ward found that *Aspergillus 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 *Aspergillus 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 *Aspergillus 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 degree Celsius and pH3.5 were optimum for itaconic acid production. Moreover, they suggested that the most suitable nitrogen source for itaconic acid production was peptone. Riscaldati et al. investigated effect of pH and stirring rate on itaconic acid production by *Aspergillus terreus*. *Aspergillus 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 *Aspergillus terreus* by mutagenesis were also investigated. 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. (Yahiro et al. 1995) In 2006 Dwiarti et al. reported that *Aspergillus terreus* TN 484-M1 reached 0.34 g itaconic acid/ g substrate when sago starch hydrolysate used as carbon source. 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.

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 *Aspergillus terreus* NRRL1960.

Materials & Method

Microorganism and medium compositions

Aspergillus 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 7thday agar plate and

suspended into the sterile DI water. The spore concentration was determined using a hemacytometer. 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 recipe were tested for suitable growth and acid production. All recipes were listed below.

Growth medium (GM)

GM1 (Yahiro et al., 1995)

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

GM2 (Riscaldati et al., 2000)

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 (Ju et al., 1986)

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 (Pfeifer et al., 1952)

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 (Pfeifer et al., 1952)

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

PM2 (Ju et al., 1986)

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 (Lockwood et al., 1952)

Glucose	100	g/L
$(NH_4)_2SO_4$	3.0	g/L
$MgSO_4 \cdot 7H2O$	0.5	g/L
$CaSO_4$	3	g/L

PM4 (Riscaldati et al., 2000)

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

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.

Free cell fermentation in the 5 L stirred bioreactor

Similarly to the flask culture, the fermentation optimization was performed in the bioreactor scale. The media optimized in flask culture 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.

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 free cell fermentation described above. 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.

Analytical methods

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:

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

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: 300mm × 7.8mm) 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.

Results and Discussion

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

Medium and pH optimization for initiating high cell density of *A. terreus*

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. 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 growth medium and the pH were optimized.

First step of experiment, the medium compositions were studied. In this study, the medium compositions were selected from previous study in the topic of itaconic acid production from *A. terreus*. Five growth medium recipes were selected in the study. In growth phase, *A. terreus* NRRL1960 were cultured in 250mL Erlenmeyer flask containing 50 mL growth medium. The temperature was controlled at 30 C and shaken at 150 rpm. Tables 1-5 show glucose consumption and cell growth obtained from different media.

Table 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 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)	$Y_{x/s}$ (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 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)	$Y_{x/s}$ (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 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)	$Y_{x/s}$ (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 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)	$Y_{x/s}$ (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

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.

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 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.

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.

Table 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.

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

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 medium, the optimized pH was initially set at 2 as reported in the previous literatures. The culture was incubated for 7 days at 30 $^{\circ}$C. Table 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.

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. Table 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.

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.

Immobilization of *A. terreus* on a cotton cloth

In many 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 witht 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 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.

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

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

Summary

It was found that 2 phase fermentation was effectively used for promoting growth and itaconic acid production. Compared with the immobilized cells on the cotton cloth, the free cell pellets gave the high glucose consumption rate and itaconic acid production. Further optimization in the bioreactor scale is on going.

Table 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

Table 8 Effect of rotational speed on itaconic acid production by *A. terreus* cultivated in PM1 at 30 °C and pH 2 for 7 days

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

Table 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

Bibliography

Batti, M.A. 1964. Process for the production of Itaconic acid. U.S. Patent. 3162582

Dwiarti, L., Otsuka, M., Miura, S., Yaguchi, M. and Okabe, M. 2007. Itaconic acid production using sago starch hydrolysate by *Aspergillus terreus* TN484-M1. *Bioresource technology*. 98, 3329-3337.

Eimhjellen, K.E. and Larsen, H. 1954. The mechanism of itaconic acid by *Aspergillus terreus*. *Biochemical Journal.* 60, 139-147.

Elnaghy, M.A. and Megalla, S.E. 1975. Itaconic production by a local strain of *Aspergillus terreus*. *European journal of applied microbiology and biotechnology*. 2, 159-172.

Ju, N. and Wang, S.S. 1986. Continuous production of itaconic acid by *Aspergillus terreus* immobilized in a porous disk bioreactor. *Applied Microbiology and Biotechnology*. 23, 311-314.

Kuenz, A., Gallenmuller, Y., Willke, T. and Vorlop, K.D. 2012. Microbial production of itaconic acid developing a stable platform for high product concentrations. *Applied Microbiology and Biotechnology (in press)*.

Nelson, G.E.N., Traufler, D.H., Kelley, S.E. and Lockwood, L.B. 1952. Production of itaconic acid by *Aspergillus terreus* in 20-liter fermenters. *Industrial and Engineering Chemistry*. 44, 1166-1168.

Pfeifer, V.E., Vojnovich, C. and Heger, E.N. 1952 itaconic acid by fermentation with *Aspergillus terreus*. *Industrial and Engineering Chemistry*. 44, 2975-2980.

Reddy, C.S.K. and Singh, R.P. 2002. Enhanced production of itaconic acid from corn starch and market refuse fruits by genetically manipulated *Aspergillus terreus* SKR10. *Bioresource Technology*. 85, 69-71.

Riscaldati, E., Moresi, M., Federici, F. and Petruccioli, M. 2000. Effect of pH and stirring rate on itaconate production by *Aspergillus terreus*. *Journal of Biotechnology*. 83, 219-230.

Shimi, I.R. and Nour El Dein, M.S. 1962. Biosynthesis of itaconic acid by *Aspergillus terreus*. *Archiv fur Mikrobiology*. 44, 181-188.

Winskill, N. 1983. Tricarboxylic acid cycle activity in relation to itaconic acid biosynthesis by *Aspergillus terreus*. *Journal of General Microbiology*. 129, 2877-2883.

ประวัติผู้วิจัย

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   เวลาที่ใช้ทำวิจัย ชั่วโมง):สัปดาห์(12
4. หน่วยงานและสถานที่อยู่ที่ติดต่อได้สะดวก พร้อมหมายเลขโทรศัพท์ โทรสาร และไปรษณีย์อิเล็กทรอนิกส์) e-mail)
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5. ประวัติการศึกษา

มหาวิทยาลัย	ปริญญา	สาขาวิชา	ปี พที่ได้รับ .ศ.
The Ohio State University	Ph.D.	Chemical Engineering	2548
Asian Institute of Technology	M.Eng.	Bioprocess Technology	2541
จุฬาลงกรณ์มหาวิทยาลัย	B.Eng.	วิศวกรรมเคมี	2538

6. สาขาวิชาการที่มีความชำนาญพิเศษ) แตกต่างจากวุฒิการศึกษา (ระบุสาขาวิชาการ
   Biotechnology, Bioengineering and Bioprocessing: emphasizing on development of novel fermentation and bioseparation processes to produce value-added products such as organic acid and biopolymer from low-value substrates locally available.
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละผลงานวิจัย
   7.1. ผู้อำนวยการแผนงานวิจัย: ชื่อแผนงานวิจัย
   -
   7.2. หัวหน้าโครงการวิจัย: ชื่อโครงการวิจัย
   - Improved lactic acid fermentation by immobilization of *Rhizopus oryzae* NRRL395 on fibrous matrices: I. strain improvement. (Ratchadapiseksomphot Endowment Fund – Seed Money Phase I; 120,000 Baht; 2005-2006)
   - Improved L-lactic acid production by Rhizopus oryzae by alteration of metabolic pathway using promoter and/or inhibitor. (Ratchadapiseksomphot Endowment Fund – Seed Money Phase II; 120,000 Baht; 2007-2009)
   - L(+)-lactic acid fermentation from cassava pulp by *Rhizopus oryzae*. (Thailand Research Fund – Seed Money; 480,000 Baht; 2007-2009)
   - Enhanced treatment of hemorrhage from open wounds by biodegradable copolymer wound dressing device containing Thai medicinal plant extract. (Rapier-Behr Co., Ltd., Singapore; 1,811,200 Baht; 2008-2010)
   - Enhanced L(+)-lactic acid production by inhibition of alcohol dehydrogenase in *Rhizopus oryzae*. (TRF-MAG Window II Co-funding; 200,000 Baht; 2008-2010)

- Screening of Thai native bacteria that give high lactic acid production. (Musashino Chemical Laboratory, Ltd., Japan; 1,985,000 Baht; 2009-2010)
- Stopping hemorrhage by biodegradable copolymer wound dressing (prelim). (National Research University Project of CHE and Ratchadapiseksomphot Endowment Fund (AM007I); 320,000 Baht; 2010-2011)
- Facility use and experimental support regarding ethanol fermentation using a sugarcane molasses material. (Iwata Chemical Co., Ltd., Japan; 750,000 Baht; 2010)
- Development of alternated technology for production of polylactic acid feedstock (National Research University Project of Commission on Higher Education and Ratchadapiseksomphot Endowment Fund (AM1026A) – Phase I; 300,000 Baht; 2010-2011)
- Pilot scale production of L-lactic acid from raw cassava pulp by *Rhizopus oryzae*. (National Research Council of Thailand via Ratchadapiseksomphot Endowment Fund; 400,000 Baht; 2010-2011(
- Pilot scale production of L-lactic acid from cassava pulp hydrolysate by *Rhizopus oryzae*. (National Research Council of Thailand; 700,000 Baht; 2011-2012)
- Technological development for biosuccinic acid production from renewable feedstocks available in South East Asia. (PTT Global Chemical Public Company Limited; 1,200,000 Baht; 2011-2014)
- Development of alternated technology for production of polylactic acid feedstock. (National Research University Project of Commission on Higher Education and Ratchadapiseksomphot Endowment Fund (AM1026A-55) – Phase II; 210,000 Baht; 2011-2012)
- Development of alternated technology for production of polylactic acid feedstock. (National Research University Project of Commission on Higher Education and Ratchadapiseksomphot Endowment Fund (AM1026A-56) – Phase III; 400,000 Baht; 2012-2013)
- D – lactic acid bacterial selection and its optimal fermentation condition. (Integrated Innovation Academic Center: IIAC Chulalongkorn University Centenary Academic Development Project (CU56 – AM05); 274,000 Baht; 2012 – 2013.
- Screening thermophilic lactic acid producing bacteria and developing a bench-scale fermentation process for industrial polylactic acid production. (PTT Global Chemical Public Company Limited; 3,491,000 Baht; 2012-2014)
- Development of itaconic acid production technology for bioplastic using immobilized *Aspergillus terreus* on natural fiber in the static bed bioreactor. (National Research Council of Thailand; 750,000 Baht; 2013-2015)
- ATP and NADH/NAD^+ manipulation for increased itaconic acid in *Aspergillus terreus* NRRL1960 (Ratchadapiseksomphot Endowment Fund (RES560530182-AM); 750,000 Baht; 2013-2015)
- โครงการวิจัยและพัฒนาเทคโนโลยีชีวภาพสำหรับการผลิตสารประกอบเคมีขั้นพื้นฐานจากวัตถุดิบธรรมชาติ (PTT Global Chemical Public Company Limited; 400,000 Baht: 2014-2015)

7.3. งานวิจัยที่ทำเสร็จแล้ว: ชื่อผลงานวิจัย ปีที่พิมพ์ การเผยแพร่ และแหล่งทุน) อาจมากกว่า 1 เรื่อง(

- Thongchul, N. and Yang, S.-T., Controlling biofilm growth and lactic acid production by *Rhizopus oryzae* in a rotating fibrous bed bioreactor: effects of dissolved oxygen, rotational speed, and urea concentration. *Journal of the Chinese Institute of Chemical Engineers*, 2006, 37 1-13.
- Kulpreecha, S., Boonruangthavorn, A., Meksiriporn, B., and Thongchul, N., Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*. *Journal of Bioscience and Bioengineering*, 2009, 107 240-245.
- Boonkong, W., Sangvanich, P., Petsom, A., and Thongchul, N., Comparison of ion exchanger and in-house electrodialysis unit for recovery of L-lactic acid from fungal fermentation broth. *Chemical Engineering&Technology*, 2009, 32 1542-1549.
- Thongchul, N., Navankasattusas, S., and Yang, S.-T., Production of lactic acid and ethanol by *Rhizopus oryzae* integrated with cassava pulp hydrolysis. *Bioprocess and Biosystems Engineering*, 2010, 33 407-416.
- Chotisubha-anandha, N., Thitiprasert, S., Tolieng, V., and Thongchul, N., Improved oxygen transfer and increased L-lactic acid production by morphology control of *Rhizopus oryzae* in a static bed bioreactor. *Bioprocess and Biosystems Engineering*, 2011, 34 163-172.
- Vallapa, N., Wiarachai, O., Thongchul, N., Pan, J., Tangpasuthadol, V., Kiatkamjornwong, S., and Hoven, V.P., Enhancing antibacterial activity of chitosan surface by heterogeneous quarternization. *Carbohydrate Polymers*, 2011, 83 868-875.
- Thitiprasert, S., Sooksai, S., and Thongchul, N., *In vivo* regulation of alcohol dehydrogenase and lactate dehydrogenase in *Rhizopus oryzae* to improve L-lactic acid fermentation. *Applied Biochemistry and Biotechnology*, 2011, 164 1305-1322.
- Wiarachai, O., Thongchul, N., Kiatkamjornwong, S., and Hoven, V.P., Surface-quarternized chitosan particles as an alternative and effective organic antibacterial material. *Colloids and Surfaces B: Biointerfaces*, 2012, 92 121-129.
- Sriputtirat, S., Boonkong, W., Pengpreecha, S., Petsom, A., and Thongchul, N., Low molecular weight poly(lactide-co-caprolactone) for tissue adhesion and tetracycline hydrocholoride controlled release in wound management. *Advances in Chemical Engineering and Science*, 2012, 2 15-27.
- Kommanee, J., Tanasupawat, S., Yukphan, P., Thongchul, N., Moonmangmee, D., and Yamada, Y., Identification of *Acetobacter* strains isolated in Thailand based the phenotypic, chemotaxonomic, and molecular characterizations. *Science Asia*, 2012, 38 44-53.
- Kommanee, J., Tanasupawat, S., Yukphan, P., Moonmangmee, D., Thongchul, N., and Yamada, Y., Identification and oxidation products of Gluconobacter strains isolated from fruits and flowers in Thailand. *International Journal of Biology*, 2012, 4 69-80.
- Phrueksawan, P., Kulpreecha, S., Sooksai, S., and Thongchul, N., Direct fermentation of L(+)-lactic acid from cassava pulp by solid state culture of *Rhizopus oryzae*. *Bioprocess and Biosystems Engineering*, 2012, 35 1429-1436.
- Noppakundilograt, S., Sonjaipanich, K., Thongchul, N., and Kiatkamjornwong, S., Syntheses, characterization and antibacterial activity of chitosan grafted hydrogels and associated mica-

containing nanocomposite hydrogels. *Journal of Applied Polymer Science*, 2013, 127 4927-4938.

- Boonkong, W., Petsom, A., and Thongchul, N., Rapidly stopping hemorrhage by enhancing blood clotting at an opened wound using chitosan/polylactic acid/polycaprolactone wound dressing device. *Journal of Materials Science – Materials in Medicine*, 2013, 24 1581-1593.
- Kanjanachumpol, P., Kulpreecha, S., Tolieng, V., and Thongchul, N., Enhancing polyhydroxybutyrate production from high cell density fed-batch fermentation of *Bacillus megaterium* BA-019. *Bioprocess and Biosystems Engineering*, 2013, 36 1463-1474.
- Thamacharoensuk, T., Thongchul, N., Taweechotipatr, M., Tolieng, V., Kodama, K., and Tanasupawat, S., Screening and characterization of lactic acid bacteria from animal faeces for probiotic properties. *Thai Journal of Veterinary Medicine*, 2013, 43 541-551.
- Phulkerd, P., Thongchul, N., Bunyakiat, K., and Petsom, A., Coal water slurry using dispersant synthesized from cashew nut shell liquid (CNSL). *Fuel Processing Technology*, 2014, 119 256-262.
- Thitiprasert, S., Sooksai, S., and Thongchul, N., 1,2-diazole and 2,2,2-trifluoroethanol and their regulatory effects on ethanol and lactic acid formation in the living culture of *Rhizopus oryzae*. *Applied Biochemistry and Biotechnology*, 2014, 172 1673-1686.
- Thitiprasert, S., Songserm, P., Boonkong, W., Sooksai, S., Kodama, K., and Thongchul, N., Manipulating pyruvate decarboxylase by addition of enzyme regulators during fermentation of *Rhizopus oryzae* to enhance lactic acid production. *Applied Biochemistry and Biotechnology*, 2014, 174 1795-1809.
- Thamacharoensuk, T., Kitahara, M., Ohkuma, M., Thongchul, N., and Tanasupawat, S.*, *Sporolactobacillus spathodeae* sp. nov., two spore-forming lactic acid bacteria isolated from tree barks in Thailand. International Journal of Systematic and Evolutionary Microbiology (in press).

7.4. งานวิจัยที่กำลังทำ: ชื่อข้อเสนอการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด

- Development of itaconic acid production technology for bioplastic using immobilized *Aspergillus terreus* on natural fiber in the static bed bioreactor. (National Research Council of Thailand; 750,000 Baht; 2013-2015) ดำเนินการวิจัยลุล่วงแล้วประมาณร้อยละ 50
- ATP and NADH/NAD^+ manipulation for increased itaconic acid in *Aspergillus terreus* NRRL1960 (Ratchadapiseksomphot Endowment Fund (RES560530182-AM); 750,000 Baht; 2013-2015) ดำเนินการวิจัยลุล่วงแล้วประมาณร้อยละ 50
- โครงการวิจัยและพัฒนาเทคโนโลยีชีวภาพสำหรับการผลิตสารประกอบเคมีขั้นพื้นฐานจากวัตถุดิบธรรมชาติ (PTTGC; 400,000 Baht; 2014-2015) ดำเนินการวิจัยลุล่วงแล้วประมาณร้อยละ 80
- Optimized conditions for D-lactic acid fermentation by *Sporolactobacillus laevolacticus* in the stirred fermentor (โครงการยุทธศาสตร์การวิจัยเชิงลึก กองทุนรัชดาภิเษกสมโภช คลัสเตอร์วัสดุขั้นสูง (Advanced Materials); 2,000,000 Baht; 2014-2015) ดำเนินการวิจัยลุล่วงแล้วประมาณร้อยละ 50

สัญญาเลขที่ GRB_APS_๔๕_๕๗_๖๑_๐๓

โครงการวิจัย เรื่อง การพัฒนาเทคโนโลยีการผลิตกรดอิทาโคนิคเพื่อการผลิตพลาสติกชีวภาพโดยเซลล์ตรึง

ของ *Aspergillus terreus* บนเส้นใยธรรมชาติในถังปฏิกรณ์ชีวภาพแบบเบดสถิต ปีที่1

รายงานการรับ-จ่ายเงิน

	ประมาณการ	งบที่เกิดขึ้นจริง
รายได้		
เงินอุดหนุนงบประมาณแผ่นดิน	250,000.00	250,000.00
รวมรายได้	250,000.00	250,000.00
รายจ่าย		
หมวดค่าจ้างชั่วคราว	-	-
หมวดค่าตอบแทน	25,000.00	25,000.00
หมวดค่าใช้สอย	110,000.00	18,289.50
หมวดค่าวัสดุ	115,000.00	206,745.74
หมวดค่าครุภัณฑ์ (ถ้ามี)	-	-
รวมรายจ่าย	250,000.00	250,035.24

รายรับสูงกว่ารายจ่าย นำส่งส่วนการคลังจุฬาลงกรณ์มหาวิทยาลัย (เอกสารแนบ)	0.00
ดอกเบี้ย (ถ้ามี)	0.00
รวมจำนวนเงินที่นำส่งส่วนการคลังทั้งสิ้น	0.00

ขอรับรองว่ารายงานการรับ-จ่ายเงินข้างต้นเป็นความจริงทุกประการ

(ผู้ช่วยศาสตราจารย์ ดร. ณัฏฐา ทองจุล)

หัวหน้าโครงการวิจัย

----------/----------/----------

หมายเหตุ: รายงานตลอดโครงการเมื่อสิ้นสุดโครงการ/ปิดโครงการ