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

Aspergillus terreus was reported as the promising fungal strain for itaconic acid; however, the commercial production suffers from the low yield. Low production yield was claimed as the result of completing TCA cycle towards biomass synthesis while under limiting phosphate and nitrogen, TCA cycle was somewhat shunted and consequently the metabolite fluxes move towards itaconic acid production route. By regulating enzymes in TCA cycle, it is believed that itaconic acid production can be improved. One of the key responsible enzymes involved in itaconic acid production was triggered in this study. Pyruvate carboxylase was allosterically inhibited by L-aspartate. The presence of 10 mM L-aspartate in the production medium directly repressed PC expression in the living *A. terreus* whilst the limited malate flux regulated the malate/citrate antiporters resulting in the increasing cis-aconitate decarboxylase activity to simultaneously convert cis-aconitate, citrate isomer, into itaconic acid. The transport of cis-aconitate via the antiporters induced citrate synthase and 6-phosphofructo-1-kinase activities in response to balance the fluxes of TCA intermediates. Successively, itaconic acid production yield and final concentration could be improved by 8.33% and 60.32%, respectively compared to those obtained from the control fermentation with the shortened lag time to produce itaconic acid during the production phase.

บทคัดย่อ

มีการรายงานว่า ราสายพันธุ์ *Aspergillus terreus* เป็นราที่มีศักยภาพในการหมักกรดอิทาโคนิค อย่างไรก็ตาม ในกระบวนการหมักมักประสบปัญหาผลผลิตกรดที่ได้ต่ำ ซึ่งอัตราการผลิตกรดอิทาโคนิคที่ต่ำนั้นสามารถอธิบายได้จากการเกิดเมแทบอลิซึมภายในเซลล์ผ่านวัฏจักรเครบส์แบบสมบูรณ์ ซึ่งในการผลิตกรดอิทาโคนิคนั้น ฟลักซ์ของเมแทบอไลท์บางส่วนจะต้องถูกผันออกจากวัฏจักรเครบส์ ซึ่งปรากฏการณ์ดังกล่าวจะเกิดขึ้นในสภาพการหมักที่มีแหล่งฟอสเฟตและไนโตรเจนในปริมาณจำกัด ดังนั้น จึงมีสมมติฐานที่ว่าประสิทธิภาพของการหมักกรดอิทาโคนิคจะเพิ่มขึ้นได้ถ้าหากสามารถควบคุมการทำงานของเอนไซม์ที่อยู่ในวัฏจักรเครบส์ได้ ในโครงการวิจัยนี้ มุ่งศึกษากลไกการทำงานของไพรูเวทคาร์บอซิเลสซึ่งเป็นหนึ่งในเอนไซม์ในวัฏจักรเครบส์ที่มีส่วนเกี่ยวข้องต่อการผลิตกรดอิทาโคนิค โดยเอนไซม์นี้จะถูกยับยั้งโดย แอล-แอสพาร์เทตที่เติมลงในอาหารเลี้ยงเชื้อที่ความเข้มข้น 10 มิลลิโมลาร์ในช่วงการผลิตกรด ผลของการยับยั้งไพรูเวทคาร์บอซิเลสแสดงออกต่อการทำงานของแอนติพอร์เตอร์ (มาเลท/ซิเทรต) ซึ่งส่งผลต่อเนื่องให้การทำงานของเอนไซม์ซิส-อะโคนิเทตดีคาร์บอซิเลสมีประสิทธิภาพที่ดีขึ้น ทำให้การเกิดไอโซเมอไรเซชั่นของซิเทรตไอโซเมอร์เกิดขึ้นอย่างต่อเนื่อง ซึ่งท้ายที่สุดแล้วส่งผลให้เกิดการเพิ่มผลผลิตของกรดอิทาโคนิคในที่สุด และเนื่องจากประสิทธิภาพในการแลกเปลี่ยนซิส-อะโคนิเทตผ่านทางแอนติพอร์เตอร์สูงขึ้น จึงส่งผลให้การทำงานของเอนไซม์ซิเทรตซินเทส และเอนไซม์ 6-ฟอสโฟฟรักโท-ไคเนสเพิ่มขึ้นตามกัน ทั้งนี้เพื่อเป็นการรักษาสมดุลของฟลักซ์ภายในวัฏจักรเครบส์ ซึ่งท้ายที่สุดส่งผลให้อัตราผลผลิตและความเข้มข้นสุดท้ายของกรดอิทาโคนิคเพิ่มสูงขึ้นเป็น 8.33 เปอร์เซ็นต์ และ 60.32 เปอร์เซ็นต์ตามลำดับ เมื่อนำไปเปรียบเทียบกับค่าที่ได้จากการหมักโดยวิธีเดิม อีกทั้งช่วงเวลาที่ใช้ในการหมักยังสั้นขึ้นอีกด้วย

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Introduction

Itaconic acid, or methylene succinic acid ($C_5H_6O_4$), is an unsaturated 5-carbon dicarboxylic acid. It is a naturally occurring nontoxic compound which is readily compostable. Itaconic acid plays a crucial role as a monomer or an additive in many industrial applications including synthetic resins, synthetic fibers, artificial glass, detergents, and paints [1]. Most importantly, from the US Department of Energy (DOE) report, itaconic acid has been classified as one of the top 12 chemical building blocks derived from biomass that can be used as a precursor in the production of various high-value bio-based chemicals or materials [2].

To date, itaconic acid is commercially produced via *Aspergillus terreus* fermentation which occurred toward the glycolytic pathway and the early step of the oxidative route in TCA cycle [3]. Fungal fermentation process to produce itaconic acid usually suffers from low product yield and long production time. As a result, the production capacity cannot guarantee the increasing market demand to substitute the petroleum-based feedstock in the near future. Several optimization techniques to develop the conditions that favor itaconic acid have been applied during fermentation but to date none of them did not well accomplished. Though the patents from the 1960s showed very good itaconic acid production processes, the results varied a lot by the slightly change in trace elements present in the medium to the bioreactor geometries. Even the results obtained from the same strains were fluctuated between the shaking flasks and different fermentor geometries (stirred tank vs. air-lift) [4]. In 2012, Kuenz et al. reported itaconic acid production using different bioreactors by *A. terreus* strains [1]. From the previous literatures, reproducibility seems to be a major problem in the production process. Recent progress argued that overproduction of itaconic acid required an interaction of several factors including a nutrient limitation for uncoupled glycolysis and

oxidative phosphorylation, a possibility to decrease the high energy level, a specific transporter system to transport TCA intermediates into the cytosol, and CAD to decarboxylate cis-aconitate to itaconic acid. However, the exact regulation of itaconic synthesis is not yet fully understood [5].

It was claimed that ATP plays a key role in controlling the metabolic fluxes in the glycolytic pathway and TCA cycle. ATP is the major energy molecule required in many metabolic reactions to produce the value added industrial products. It can serve as the substrate, product, activator and inhibitor in the metabolic network [6]. Also, in itaconic acid production, ATP helps maintain the intracellular pH near the physiological pH at 7.0 and facilitates acid transport into the surrounding environment (pH 2.0). Therefore, conserving ATP with the regulated metabolic fluxes in TCA cycle towards itaconic acid production route is the challenging way to improve its production.

To increase the availability of ATP during itaconic acid fermentation, it was hypothesized that by inhibiting pyruvate carboxylase (PC), more ATP would be available for itaconic acid production and transport. PC, a biotin-dependent carboxylase, converts 1 mole of pyruvate into 1 mole of oxaloacetate (OAA) with supplemented 1 mole of CO_2 and 1 mole of ATP input. By inhibiting PC conversion to OAA, 2 moles of ATP would be conserved for itaconic acid production and transport that eventually resulted in the improved yield and productivity. The in vitro study on fungal PC inhibition claimed that L-aspartate was an allosteric inhibitor that competitively bound to PC [7]. The activity of purified PC from the fungal cultures such as *Rhizopus arrhizus* and *Aspergillus nidulans* was inhibited when L-aspartate was added into the reaction assay [8-9]. From the previous findings, adding L-aspartate into the living culture of *A. terreus* would help decrease the activity of PC; thus, conserving ATP for itaconic acid production and TCA products transport. In this work, we reported the response of the living *A. terreus* on L-aspartate present in the fermentation

medium during the cultivation for itaconic acid production. The optimized concentration of this well-reported in vitro PC inhibitor was elucidated for its role as the direct PC regulator in the living fungal culture. Also, the change in metabolic flux when L-aspartate was added into the culture broth was reported in term of the fermentation kinetics and the activities of the key enzymes in the pathway.

Materials and methods

Itaconic acid production with the presence of pyruvate carboxylase regulator was investigated in the living culture of *A. terreus* NRRL1960 grown in the glucose-based medium in a shake flask. The effects of pyruvate carboxylase regulator on fungal growth, expression of key enzymes in the metabolic pathway, and end metabolite production were determined and compared with those obtained from the culture without the inhibitor (control).

Microorganism and inoculum preparation

Aspergillus terreus NRRL1960, a filamentous fungus producing itaconic acid kindly provided by Agricultural Research Service Culture Collection, US Department of Agriculture, Peoria, IL, USA, was used in this study. The stock culture was maintained on Czapek's agar plates and subcultured every month to maintain fungal activity. The conidiospores were collected from the 7-day culture on Czapek's agar plates incubated at 30 °C by shaving and extracting the spores with sterile water. The spore suspension was adjusted to 10^6 spores/mL by dilution with sterile DI water for inoculation.

Growing *A. terreus* in the fermentation medium containing L-aspartate

Itaconic acid fermentation by *A. terreus* consists of 2 phases. During the growth phase, the growth medium consisting of 30 g/L glucose and 5 g/L yeast extract was used to promote spore germination and initial cell growth. 0.5 mL spore suspension (10^6 spores/mL) was inoculated into a 250 mL Erlenmeyer flask containing 50 mL sterile growth medium. The initial pH during growth phase was 3.0. The culture was incubated at 30°C in a rotary shaker at 150 rpm for 48 h. At the end of the growth phase, the growth medium was replaced by the production medium containing 100 g/L Glucose, 2.36 g/L $(NH_4)_2SO_4$, 0.11 g/L KH_2PO_4, 2.08 g/L $MgSO_4 \cdot 7H_2O$, 0.13 g/L $CaCl_2.2H_2O$, 0.074 g/L NaCl, 0.2 mg/L $CuSO_4.5H_2O$, 5.5 mg/L $FeSO_4.7H_2O$, 0.7 mg/L $MnCl_2.4H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$. The pH of the production medium was initially adjusted to 2.0. The culture was incubated at the same conditions as those during the growth phase for another 192 h. During the fermentation, samples were taken every 12 h for analyses of glucose, itaconic acid, and other byproducts as well as for enzyme assays.

L-aspartate was added into the fermentation medium at different concentrations. The effects of L-aspartate on growth and metabolite production were investigated during the growth and production phases.

Product yields and volumetric productivities

Cell growth and product formation during fermentation were determined. The product yield was calculated from the ratio of product formed to glucose consumed. The volumetric productivity was defined as the total amount of product formed per volume per time.

Cell biomass

The concentration of cell biomass was determined from the cell dry weight. Cell biomass present in the fermentation broth was harvested by filtration using Whatman filter paper (No. 4). The filtered cell biomass was thoroughly rinsed with DI water before drying at 80 °C until constant weight was obtained. Cell biomass concentration present in the fermentation broth was calculated by the following equation.

$$Cell\ concentration\ (\frac{g}{L}) = \frac{Cell\ dry\ weight\ on\ filter\ paper\ (g) - dry\ weight\ of\ filter\ paper\ (g)}{Fermentation\ broth\ volume\ (L)}$$

Analysis of glucose and end products

Fermentation broth sample was centrifuged at 10,000 g for 7 min. The supernatant was kept for analyses of remaining glucose, itaconic acid, and byproducts using High Performance Liquid Chromatography (HPLC; Shimadzu model 10Ai). Before injecting the sample into the HPLC system, the sample was diluted with DDI water and filtered through the hydrophilic PTFE membrane. The particle free sample (15 μL) was automatically injected into an organic acid column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300 mm × 7.8 mm) maintained at 45 °C in a column oven. H_2SO_4 (0.4 mM) was used as an eluent at the flowrate of 0.4 mL/min. A refractive index detector was used to detect the organic compounds including glucose, itaconic acid, citric acid, cis-aconitic acid, succinic acid, oxaloacetic acid, L-malic acid, and pyruvic acid. The standard containing 0-2 g/L of each component indicated prior was injected as the reference to determine the concentrations of each compounds present in the sample. The peak area was used for the comparison basis.

Cell extraction, partial enzyme purification, and protein determination

Following the method described in Thitiprasert et al. (2014), fresh fungal mycelia from the fermentation were washed thoroughly with sterile DI water 3 times before drying between filter paper [10]. Filter-dry mycelia were weighed into 1 g and frozen at -20 °C for 1 h before grinding in an ice-cold mortar for 5 min. TrisHCl buffer (1.5 mL, 0.2 M, pH 8.0) was added into the ground mycelia. The suspension of the fungal mycelia with the glass beads was then homogenized in the cell disrupter for 16.75 min (30 s interval with 45 s break). Later, the glass beads were removed by filtration and the suspension was then centrifuged at 12,000 g for 30 min at 4 °C. The supernatant to be called later the cell extract was used for protein determination and enzyme assays. The protein content was determined by Lowry's method using a bovine serum albumin (BSA) as a standard [11].

Enzyme assays

6-phosphofructo-1-kinase (PFK) activity was determined by phosphofructokinase colorimetric assay kit (Sigma). The enzyme activity was determined from a couple enzyme reaction, in which PFK catalyzed the conversion of fructose-6-phosphate and ATP to fructose-1,6-diphosphate and ADP. ADP was later converted by the enzyme in the mixture into AMP and NADH. The reaction mixture containing 42 µL PFK assay buffer, 2 µL PFK enzyme mix, 2 µL PFK developer, 2 µL ATP and 2 µL PFK substrate was added into a 96 well plate. Then, 10 µL crude enzyme extract was added into each well. The resulting NADH reduced the colorless indicator resulting in a colorimetric product at 450 nm proportionally to the PFK activity present in the enzyme mixture. The absorbency at 450 nm of the reaction mixture was recorded every 57 s for 5 min. The average rate of the increasing absorbency at 450 nm per min was calculated. The activity of PFK was subsequently determined using the calibration plot of absorbency at 450 nm versus NADH concentration in µmol. One unit of

PFK was determined by the amount of enzyme that generated 1.0 μmol NADH in 1 min at 37 °C, pH 7.4.

Pyruvate carboxylase (PC) was determined from a couple enzyme reaction. PC catalyzed the carboxylation of pyruvate into oxaloacetate (OAA) (Eq. 1). OAA was then reacted with acetyl CoA to form citryl CoA by citrate synthase, which was subsequently hydrolyzed to citrate and CoA (Eq. 2). In the spectrophotometer, the rate-limiting reaction catalyzed by PC (Eq. 1) is coupled to the effectively irreversible chemical reaction (Eq. 3). The reaction product TNB (thionitrobenzoic acid) was the absorbing substance with intense absorption at 412 nm.

$$\text{ATP + pyruvate + } HCO_3^- = \text{ADP + phosphate + oxaloacetate} \quad (1)$$

$$\text{Acetyl-CoA + oxalacetate + } H_2O = \text{citrate + CoA-SH} \quad (2)$$

$$\text{CoA-SH + DTNB = TNB + CoA-S-S-TNB} \quad (3)$$

PC activity was determined in the reaction mixture containing 0.050 M $NaHCO_3$, 0.005 M $MgCl_2$, 0.050 mM acetyl CoA, 0.005 M pyruvate, 0.005 M ATP, 0.200 mM 5,5'-dithio-bis(2-nitrobenzoic acid), and 5 U/mL citrate synthase in 0.1 M Tris HCl buffer (pH 8.0) in a total volume of 1.0 mL. The reaction was initiated by adding 50 μL crude enzyme extract into the reaction mixture with a run time of 60 s at 30°C. The increase in absorbency at 412 nm of 5-dithio-2-nitrobenzoate (DTNB) was measured. The PC activity was determined by using the standard curve of absorbency at 412 nm versus oxaloacetate concentration in μmol. One unit of PC activity was corresponded to the formation of 1 μmol oxaloacetate in 1 min at 30 °C.

PDC activity was assayed by following the depletion of NADH at 340 nm [12]. The reaction mixture contained 25 mM sodium pyruvate, 5 mM MgCl2, 0.2 mM thiamine pyrophosphate (TPP), 11 U/mL alcohol dehydrogenase, and 0.15 mM NADH in 100 mM MES buffer (pH 6.5) in a total volume of 1.2 mL. The reaction was started by adding diluted

enzyme sample in crude extract and the absorbency at 340 nm was recorded every 57 s for 5 min at 30 °C. The average rate of the increase in absorbency at 340 nm per min was determined. PDC activity was monitored at 340 nm corresponding to the amount of NADH (in μmol) oxidized per minute using the calibration plot of absorbency at 340 nm versus NADH concentration (μmol).

Cis-aconitate decarboxylase (CAD) activity was assayed by Bently method [13]. Crude enzyme extract (0.1 mL) was incubated with 0.4 mL cis-aconitic acid solution (to the final concentration of 8.0 mM) and 2.5 mL sodium phosphate (0.2 M, pH 6.2) for 10 min at 37 °C. The reaction was terminated by adding 0.1 mL HCl (12.0 M). The released itaconic acid was analyzed by HPLC using the similar method described earlier.

Citrate synthase (CS) was assayed by DTNB method [14]. The reaction mixture contained 0.1 M Tris HCl buffer (pH 8.0), 0.2 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.05 mM oxaloacetate, and 0.05 mM acetyl CoA in a total volume of 1.0 mL. The reaction was started by adding 50 μL crude enzyme extract into the reaction mixture. The absorbance at 340 nm was measured every 57 s for 5 min at 30 °C then the average rate of the increase in absorbency at 450 nm per min was calculated. One unit of CS was defined as 1 μmol oxaloacetate converted per minute. The amount of oxaloacetate present in the reaction mixture determine from the calibration the curve of absorbency at 412 nm versus amount of oxaloacetate (in μmol))see in Eq. 2 and 3(.

Aconitase activity was determined by aconitase activity assay kit (Sigma). The enzyme catalyzed the stereospecific and reversible isomerization of citrate to isocitrate. This resulted in a colorimetric (450 nm) product proportional to the enzymatic activity present. Before assay, crude enzyme extract was activated by adding 10 mL aconitase activation solution to 100 mL sample on ice for 1 h. Then, 10 μL activated sample was added into the reaction mixture containing 46 μL aconitase assay buffer, 2 μL aconitase substate and 2 μL

enzyme in 96 well plate at 25 °C for 60 min. After incubation, 10 µL developer was added into each well, mixed and further incubated at 25 °C for 10 min. The absorbency was measured at 450 nm. One unit of aconitase was defined as the amount of enzyme that isomerized 1.0 µmol citrate from isocitrate per minute at 25 °C , pH 7.4. The calibration plot of absorbency at 450 nm versus isocitrate concentration (µmol) was used to determine the isocitrate.

Results and discussions

Glucose-based fermentation of *A. terreus* NRRL1960

Fig. 1 shows the fermentation kinetics of *A. terreus* NRRL 1960 grown in 2 phases for itaconic acid production. Unlike other itaconic acid fermentation processes reported elsewhere in the literatures, 2-phase fermentation was aimed to achieve high cell density during the growth phase by growing *A. terreus* in the rich medium containing organic nitrogen source with the subsequent itaconic acid production by the high cell density using other medium compositions that promoted itaconic acid synthesis. During the growth phase, cell biomass was built up rapidly (8.96 g/L with 1.34 g cell per g glucose consumed and 0.19 g cell/L·h) while there was no itaconic acid produced. After 48 h the growth medium was discarded and the culture was filled up with the production medium containing $(NH_4)_2SO_4$ as the sole nitrogen source at the high C/N weight ratio of 42 to 1. Increasing cell biomass production was continuously observed at the slightly lower rate compared to that during the growth phase until 120 h (red line). When changing the medium, inorganic nitrogen ($(NH_4)_2SO_4$) with the supplemented phosphate in KH_2PO_4 in the production medium maintained the growth of *A. terreus*; however, at the lower rate compared to that cultivated in the growth medium with yeast extract. The lower growth rate confirmed the evidence of the

lower ATP regeneration due to limited phosphate pool; thus, the regeneration process occurred by substrate level phosphorylation rather than oxidative phosphorylation. At 120 h, itaconic acid was firstly observed in the fermentation broth. Later, the growth rate was dropped while glucose consumption rate remained constant and itaconic acid was produced at the constant rate (0.16 g/L·h). This finding indicated growth-limiting condition was necessary for itaconic acid synthesis by *A. terreus*. Similar growth pattern of *A. terreus* NRRL 1960 was observed by Riscaldati et al. (2000) [15]. The evidence of phosphate and nitrogen limitation led to the accumulation of itaconic acid [16]. Nonetheless, from the kinetics model non growth condition did not give the better itaconic acid production. The results show in Fig.1 and those in Riscaldati et al. (2000) reveal that the fermentation exhibited mixed growth associate product formation kinetics [15]. With the proper N/P ratio in the production medium, it is believed that itaconic acid production can be further improved in 2-phase fermentation.

Key enzymes responsible for itaconic acid synthesis

To better understand the synthesis of itaconic acid by *A. terreus* during 2-phase fermentation cultivation, the activity of the key enzymes related in itaconic acid production was assayed (Fig. 2). During the growth phase, the specific activity of PFK was approximately 2 U/mg protein while later in the production phase the PFK specific activity dramatically dropped. This was presumably due to the change in medium compositions from organic to inorganic nitrogen for slight cell growth and maintenance during the production phase. It was claimed that a univalent cations such as NH_4^+ had a role in PFK regulation as it might provoke inhibition by citrate in vivo [16-17]. Subsequently, the increasing activity of CAD (Fig. 2(a)) and the accumulation of itaconic acid (Fig. 1) were observed with the lowered PFK activity during the production phase. When growing *A. terreus* using yeast

extract under sufficient oxygen supply, glucose was rapidly metabolized into pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway. Pyruvate was then either transported to the mitochondria and later converted into acetyl CoA or carboxylated into oxaloacetate (OAA) in the cytosol [5]. Completed TCA cycle coupling with an electron transport chain (ETC) resulted in cell biomass and a large amount of ATP generation [6,18]. Changing the medium to the production medium containing a small amount of $(NH_4)_2SO_4$ as well as decreasing the initial pH to 2.0 caused the lowered PFK activity due to lower oxygen solubility at low pH and the stimulated citrate inhibition of PFK by NH_4^+. In order to balance citrate flux in the mitochondria compartment under nitrogen limitation so that glucose flux toward the glycolysis could proceed, OAA in the cytosol was converted to malate, which was transported into the mitochondria by malate/citrate antiporters. In the mitochondria, acetyl CoA and OAA were irreversibly condensed into citric acid by CS. Citric acid was then reversibly converted to isocitrate and cis-aconitate by aconitase (ACO). Cis-aconitate was transported back to the cytosol using mitochondrial tricarboxylic transporter (MTT) and then decarboxylated into itaconic acid by CAD (Fig. 3) [19]. The increasing CAD activity during the production phase was the evidence of MTT function in order to lower citrate isomer in the mitochondria thus glycolytic flux could be still driven toward pyruvate (Fig. 2 (a)). Similarly, the specific activities of PC, PDC, and CS were high during the growth phase. This confirmed the high level of the oxidative phosphorylation in *A. terreus* with sufficient oxygen supply and rich nitrogen source (Fig. 2(b)). When nitrogen was limited in the production phase, the enzyme activities dropped dramatically. This finding suggested that 2-phase fermentation of itaconic acid was successfully used to stimulate cell growth under sufficient oxygen supply and rich nitrogen source which later being used to synthesize itaconic acid under limited nitrogen.

L-aspartate as an allosteric inhibitor of PC and its role on metabolism of *A. terreus*

In this study, further growth was observed after changing the medium to the production medium (Fig. 1) while the activity of PFK, PC, PDC, and CS dramatically dropped (Fig. 2). This rather implied ATP conservation process instantaneously occurred at the beginning of the production phase when phosphate became limited. Nonetheless, as observed from the expression level of CAD, itaconic acid production started later at 120 h under both phosphate and nitrogen limitation condition [15]. To immediately initiate itaconic acid synthesis in 2-phase fermentation process after changing to the production medium, not only balancing the ratio of C/N/P in the medium is necessary, in term of the biosynthesis flux, the rate of OAA/acetyl CoA formation, citrate isomerization, and malate/cis-aconitate transport must be balanced while ATP regeneration must be also regulated (Fig. 3).

To investigate the metabolic response of *A. terreus* on medium change during 2-phase fermentation, acetyl CoA allosteric inhibitor, L-aspartate, was added into the fermentation medium. Fig. 4 shows the morphology of *A. terreus* grown in the glucose-based medium containing L-aspartate. It is clear that the morphology changed with the presence of L-aspartate. Smaller pellets were obtained when the concentration of L-aspartate was increased in the growth medium. Cell biomass production was also lower with the increasing L-aspartate concentration (Table 1). The specific activity of the key enzymes involving in biosynthesis confirmed the evidence of PC feedback inhibition by L-aspartate (Fig. 5). Lower PFK activity (Fig. 5(a)) was found with the increasing L-aspartate concentration in the growth medium. This was apparently due to L-aspartate triggered PC by binding at its allosteric site; thus, limiting PC action (Fig. 5(b)) and consequently reducing pyruvate flux towards OAA and malate. This eventually led to the reduction in biomass synthesis [7,20].

Using L-aspartate to control metabolic flux in *A. terreus* for improved itaconic acid production

Most PCs have an α_4 quaternary structure with the 4 identical subunits containing a biotin carboxylase (BC) domain, a carboxyl transferase (CT) domain, and a biotin carboxyl carrier protein (BCCP) domain. The α_4 PCs are commonly activated by acetyl CoA [21]. From the above-mentioned claim and the metabolic response of *A. terreus* to L-aspartate during the growth phase, L-aspartate was introduced into the pregrown culture of *A. terreus* during the production phase. Increasing the concentration of L-aspartate up to 10.0 mM improved itaconic acid production (Fig. 6). Not only the higher titer could be achieved, sufficient amount of L-aspartate also shortened the lag time before itaconic acid production after changing the medium. Nonetheless, excess L-aspartate somewhat caused the adverse effect on itaconic acid production when 100.0 mM was added into the production medium (Table 2). PC was strongly inhibited at 100.0 mM L-aspartate as it competitively bound at the active site preventing the conversion of pyruvate to OAA and subsequently to malate. The lowered malate pool reduced the transport fluxes between malate and cis-aconitate, the CAD substrate for itaconic acid via the malate/cis-aconitate antiporter (Fig. 3). At the same time, the large pool of acetyl CoA induced citrate formation. When high citrate concentration was present, it eventually inhibited PFK in glycolysis. This consequently slowed down the metabolism of *A. terreus* as observed from the prolonged turnover time of 120 h for itaconic acid production. As a result, itaconic acid production dropped when 100.0 mM L-aspartate was present in the production medium. From the concentration of L-aspartate studied, 10.0 mM gave the well-optimized condition that promoted itaconic acid synthesis. Approximately 1/3 shorter lag time was achieved at this concentration of L-aspartate present in the fermentation. Whilst the production yield reached up to 65% of the theoretical yield compared with that obtained from the control fermentation (60%).

The expression of PC confirmed the evidence of improved itaconic acid fermentation (Fig. 7(a)). Dramatically decreasing PC activity was observed resulting in lowered OAA in the cytosol. Subsequently, malic acid concentration was decrease in the cytosol. Malate/citrate antiporters subsequently drove the transport of cis-aconitate to the cytosol in order that malate could be transported into the mitochondria in response to the living *A. terreus* to complete TCA cycle. The presence of cis-aconitate in the cytosol induced CAD as observed from the increasing activity of this enzyme (Fig. 7(b)) and itaconic acid formation after 24 h the production phase started (Fig. 6) while the control fermentation required up to 72 h for itaconic acid production firstly observed (Fig. 6). The flux of citrate towards cis-aconitate and the transport of malate/citrate antiporters induced the activities of CS and PFK as the results of lowering citrate flux (Fig. 7(c) and (d)). As aforementioned, L-aspartate played role in allosteric inhibition of PC in the living culture of *A. terreus*. This resulted in metabolic flux shift toward itaconic acid production. The incomplete TCA cycle with the enhanced PFK activity revealed that TCA intermediates were bypassed towards itaconic acid as the result of PC inhibition and ATP conservation by the well-optimized concentration of allosteric PC inhibitor, L-aspartate, during the production phase with the limited growth of *A. terreus* [22-23].

Conclusion

In this work, we attempted to regulate *A. terreus* metabolism for enhanced itaconic acid production. PC was allosterically inhibited by L-aspartate. The results show that 10 mM L-aspartate present in the production medium of *A. terreus* repressed PC expression whilst improved CAD activity. This could be explained by the decrease in oxaloacetate (OAA) and subsequently malate fluxes induced the transport of malate and cis-aconitate, citrate isomer,

towards the antiporters as the metabolic response of the living culture of *A. terreus* to completely balance the metabolites fluxes in TCA and itaconic acid production routes. Malate transported into the mitochondria was then further converted to OAA and subsequently reacted with acetyl CoA to form citrate isomer that being transported via the antiporters to the cytosol. CAD further converted cis-aconitate, citrate isomer, into itaconic acid to balance the metabolic fluxes. This eventually resulted in the improved itaconic acid yield (0.47 g/g) of 8.33% and final concentration (34.95 g/L) of 60.32% compared to those obtained from the control fermentation (0.43 g/g and 21.80 g/L, respectively).

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Table 1 Cell biomass production during the growth phase of 2-phase fermentation of *A. terreus* for itaconic acid synthesis. L-aspartate at different concentration was added into the glucose based growth medium. The fermentation was carried out at 30 °C and 200 rpm in a shaken flask. The initial pH was 3.0.

L-aspartate conc. (mM)	Cell biomass production during the growth phase		
	Final conc. at 48 h (g/L)	Yield (g/g glucose consumed)	Productivity (g/L·h)
0 (control)	8.96±3.02	1.12	0.17
0.1	5.32±0.34	1.39	0.09
1.0	6.96±0.45	1.13	0.13
10.0	2.31±0.11	0.21	0.03

Table 2 Fermentation performance of *A. terreus* during the production phase with the presence of PC allosteric inhibitor, L-aspartate. *A. terreus* was initially pregrown in the glucose-based medium for 48 h at 30 °C, 200 rpm with the initial pH of 3.0. After 48 h, the growth medium was discarded and the culture was filled up with the production medium containing L-aspartate at different concentrations and further cultivated at the same operating conditions with the initial pH of 2.0.

Itaconic acid production	L-aspartate conc.				
	Control[c]	0.1 mM	1.0 mM	10.0 mM	100.0 mM
Final conc. (g/L)	21.80±1.17	19.45±0.04	29.14±0.65	34.95±0.70	19.49±0.10
Yield (g/g)	0.43±0.00	0.34±0.01	0.36±0.01	0.47±0.05	0.25±0.02
Productivity (g/L·h)	0.17±0.00	0.14±0.01	0.19±0.01	0.19±0.01	0.14±0.01
Time started to produced (h)[a]	120	120	120	72	120
Time reached highest concentration (h)[b]	240	240	240	240	240
Remaining glucose concentration at Time[b] (g/L)	22.34±10.89	21.92±0.07	5.64±0.87	0.00±0.00	0.99±1.40

Remarks

[a] Total time (started from growth phase of 48 h and prolonged production phase) until itaconic acid was produced.

[b] Total time (started from growth phase of 48 h and prolonged production phase) until the highest itaconic acid concentration was achieved.

[c] Fermentation without L-asparate (control fermentation)

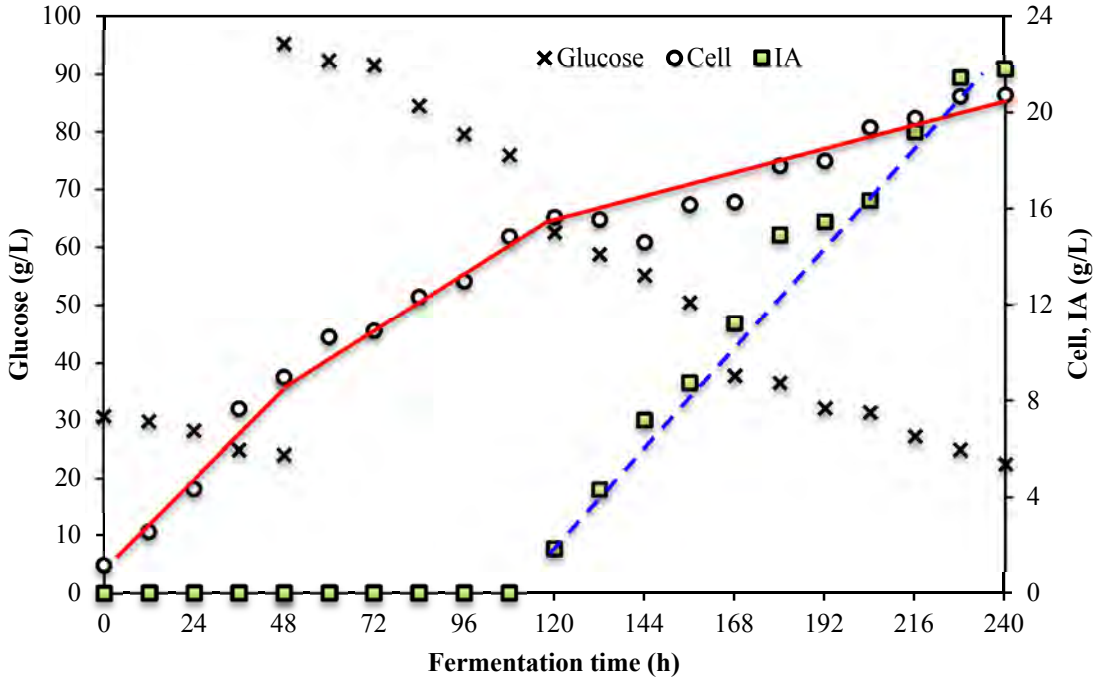
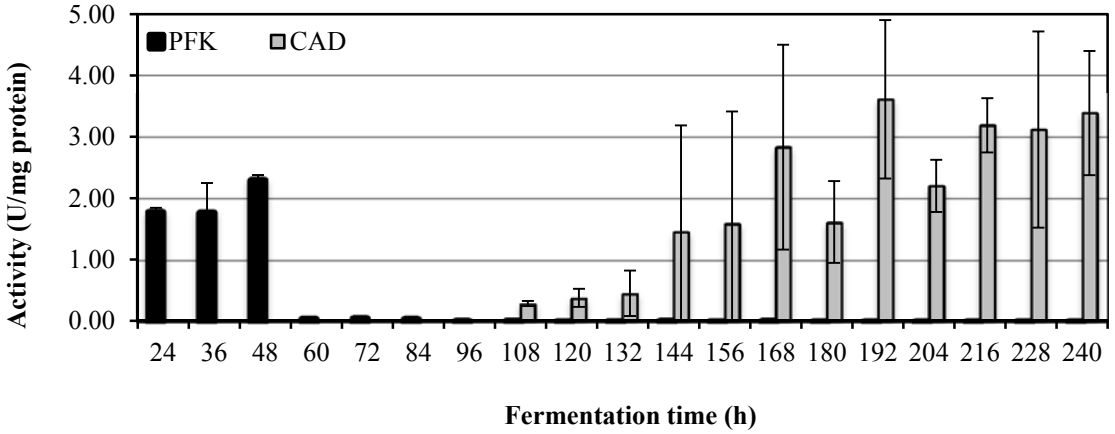


Fig. 1 Fermentation kinetics of *A. terreus* NRRL1960 grown in glucose based medium in the shake flask culture. Fermentation consisted of 2 phases. The growth phase took 48 h where yeast extract was used as a sole nitrogen source. The growth medium was replaced by the production medium containing the inorganic nitrogen source after 48 h. The fermentation was prolonged until 240 h. The red trend lines indicate 3 growth rates of *A. terreus* during cultivation. The corresponding blue trend line indicates itaconic acid production during the slow growth.

(a)



(b)

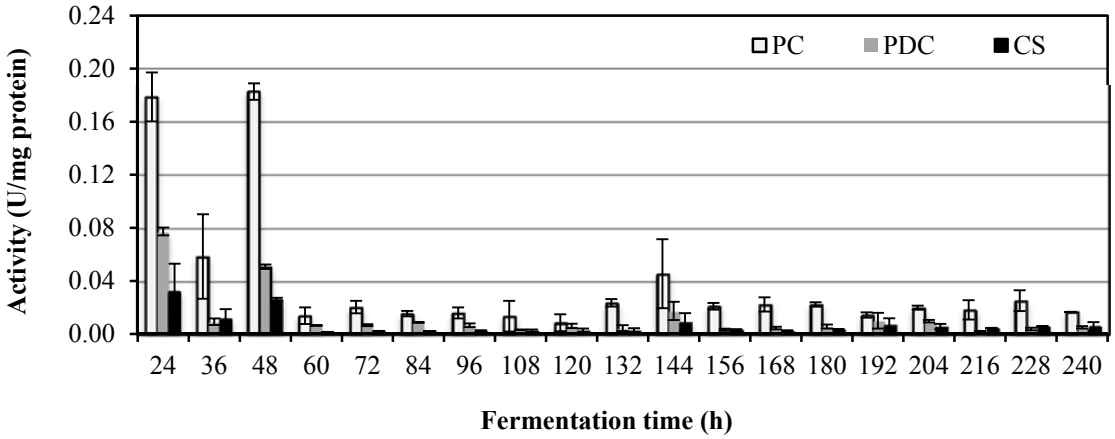


Fig. 2 Key responsible enzymes in itaconic acid production by *A. terreus*. The specific activity of these enzymes was assayed from the collected fermentation samples during cultivation of *A. terreus* in the glucose-based medium in 2-phase fermentation. Enzyme assay was carried out after 24 h cultivation when sufficient cell biomass was produced. The high expression levels of PFK and CAD were observed (more than 20x larger) (a) compared to those of PC, PDC, and CS the key intermediate enzymes in TCA cycle (b).

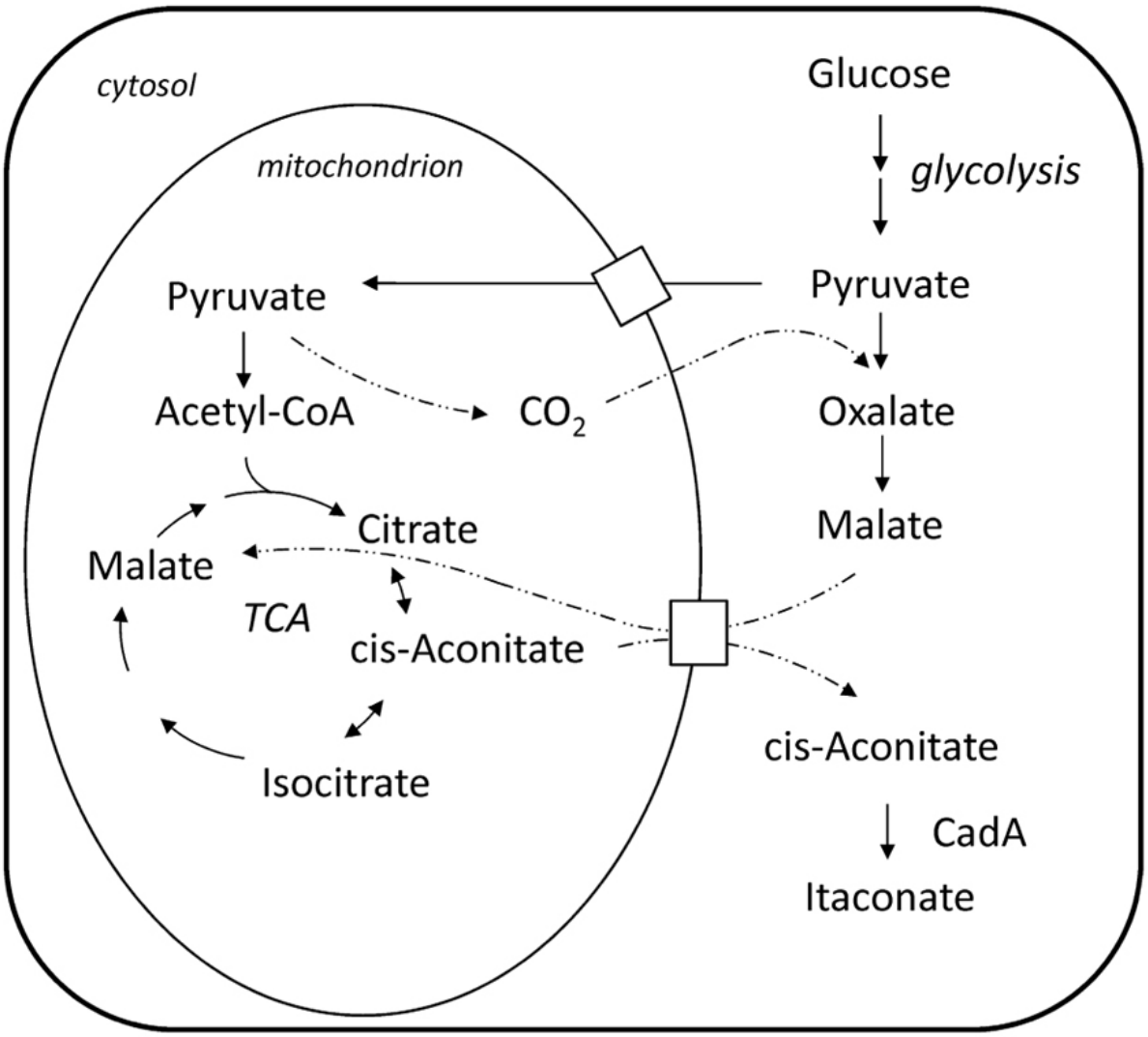


Fig. 3 Biosynthesis pathway of itaconic acid in *A. terreus*. Malate/Citrate transport during nitrogen limitation played role controlling itaconic acid synthesis. (Reprinted from Steiger et al. (2013).

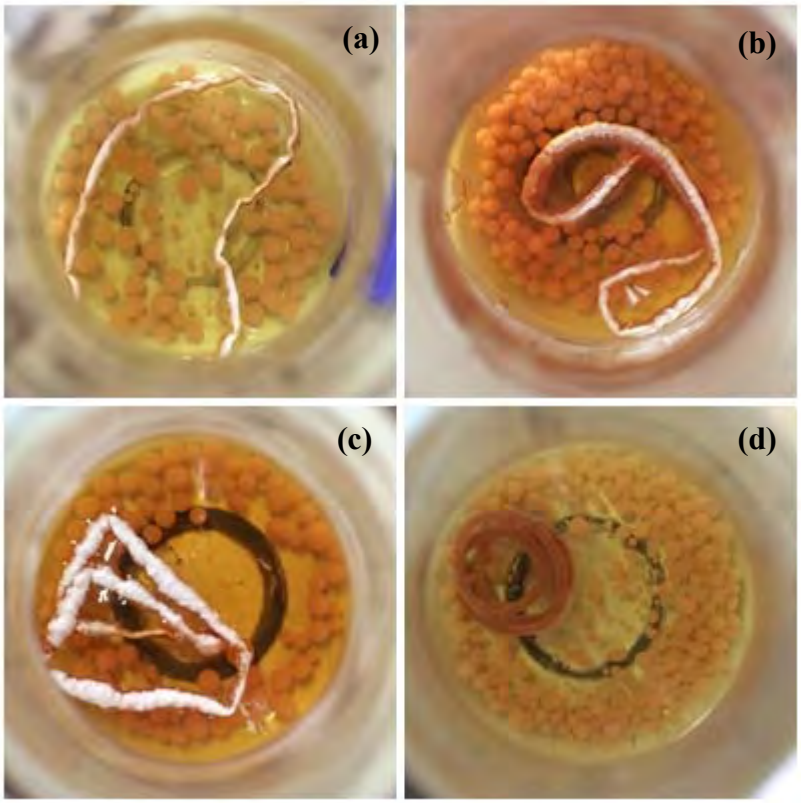
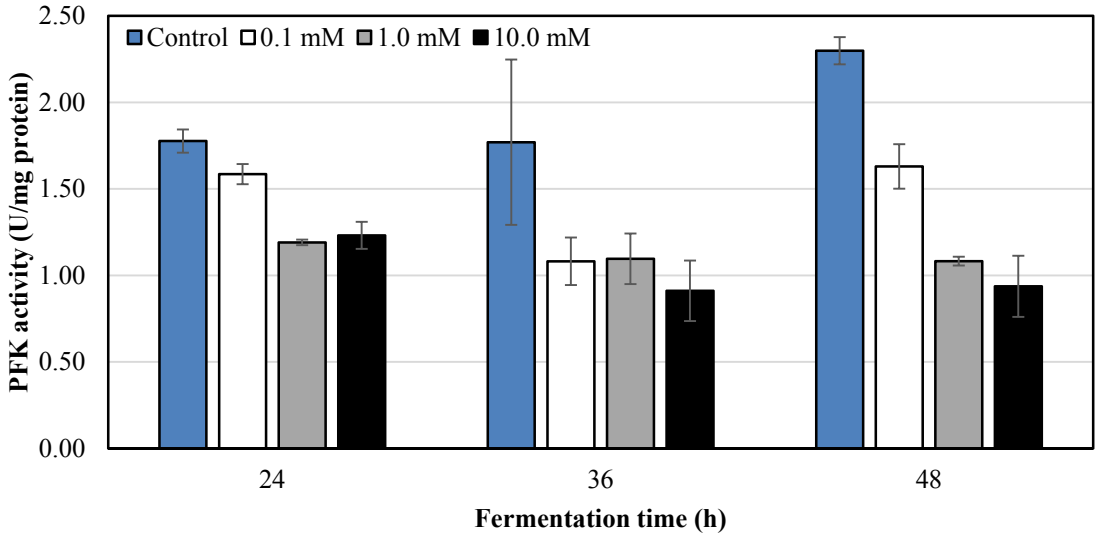


Fig. 4 Morphology of *A. terreus* cultivated in the growth medium containing L-aspartate; (a) control (no L-aspartate), (b) 0.1 mM L-aspartate, (c) 1.0 mM L-aspartate, and (d) 10.0 mM L-aspartate.

(a)



(b)

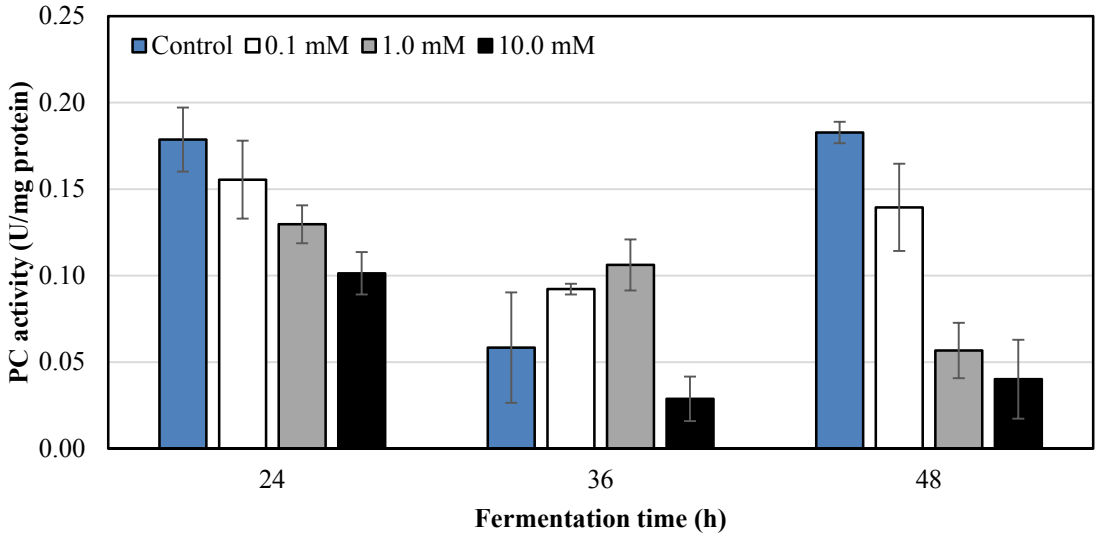


Fig. 5 The metabolic response of *A. terreus* when L-aspartate, the allosteric PC inhibitor was present during the growth phase. Key enzymes responsible for growth and ATP regeneration including PFK (a) and PC (b) were observed for their changes of activities when PC inhibitor was present.

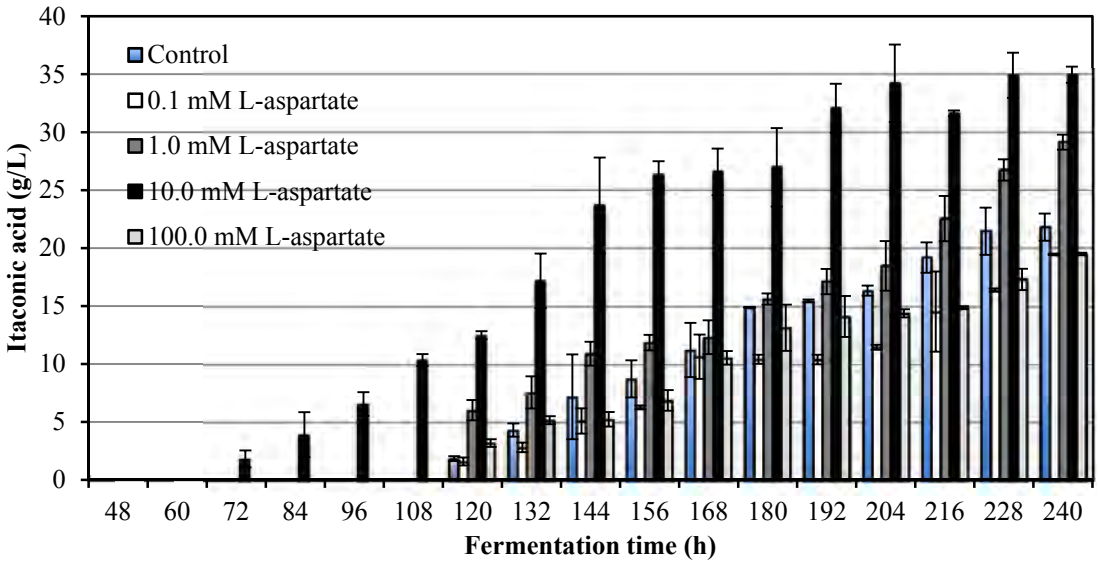
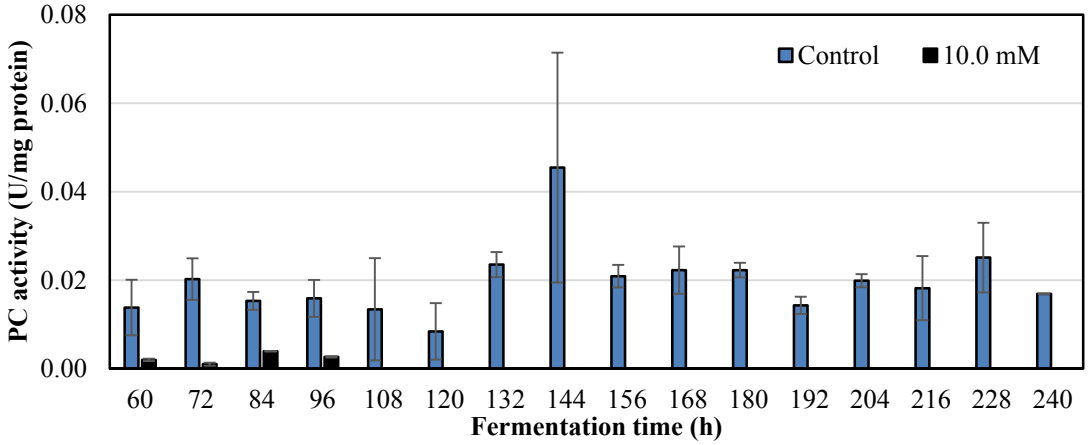
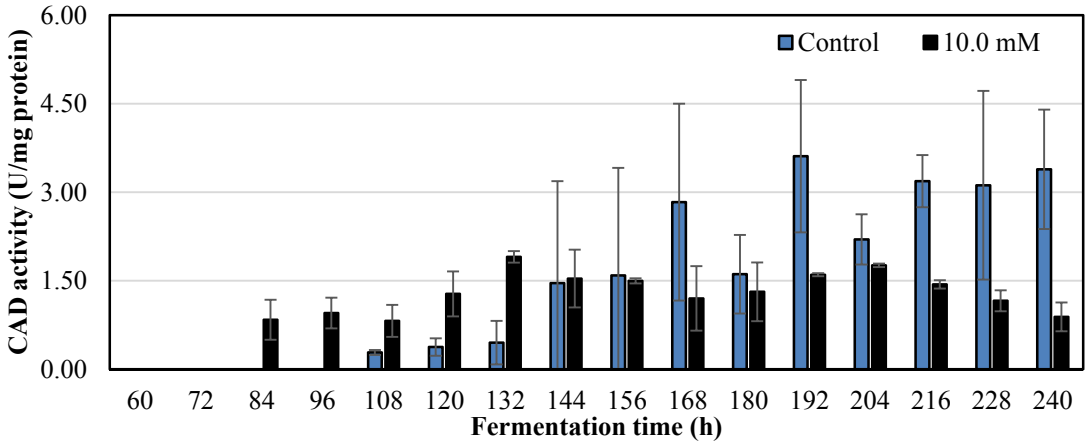


Fig. 6 Itaconic acid accumulation during the production phase of 2-phase fermentation of *A. terreus* in the glucose-based media containing an allosteric acetyl CoA inhibitor (L-aspartate) at different concentrations. *A. terreus* was pregrown in the growth medium and later it was added into the culture during the production phase. Control represented the fermentation without L-aspartate.

(a)


0.08
0.06
0.04
0.02
0.00
PC activity (U/mg protein)
Control
10.0 mM
60 72 84 96 108 120 132 144 156 168 180 192 204 216 228 240
Fermentation time (h)

(b)


6.00
4.50
3.00
1.50
0.00
CAD activity (U/mg protein)
Control
10.0 mM
60 72 84 96 108 120 132 144 156 168 180 192 204 216 228 240
Fermentation time (h)

(c)

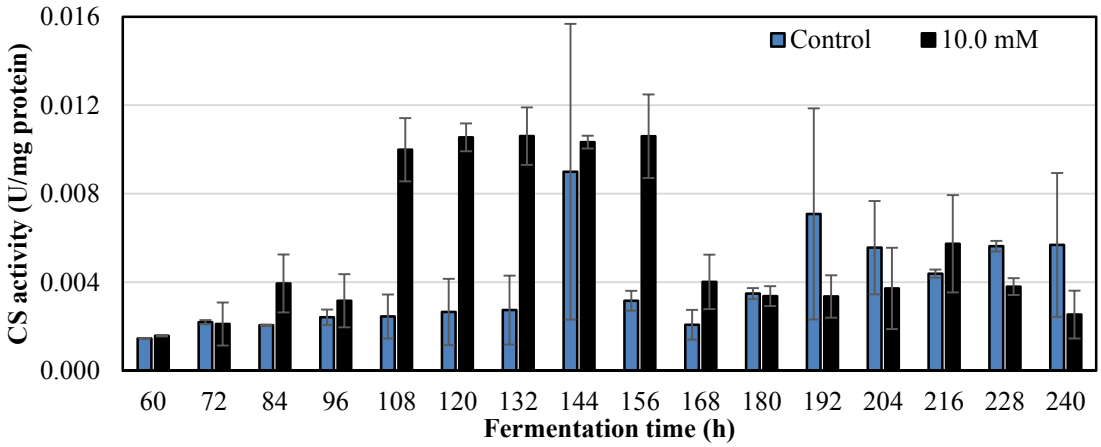

0.016
0.012
0.008
0.004
0.000
CS activity (U/mg protein)
Control
10.0 mM
60 72 84 96 108 120 132 144 156 168 180 192 204 216 228 240
Fermentation time (h)

Fig. 7 (continued)

(d)

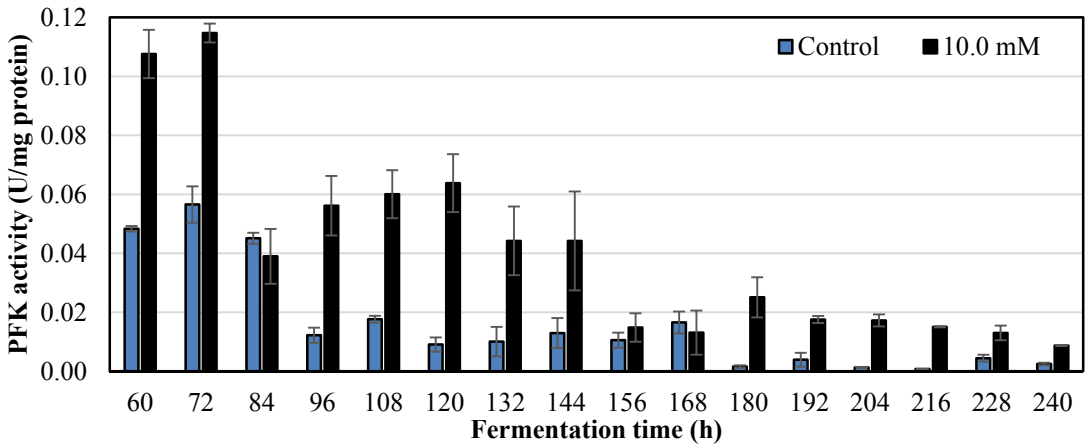


Fig. 7 Specific activities of the key responsible enzymes in itaconic acid production by *A. terreus* when L-aspartate, the allosteric PC inhibitor was present at 10.0 mM during the production phase in 2-phase fermentation.

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Asian Institute of Technology	M.Eng.	Bioprocess Technology	2541
จุฬาลงกรณ์มหาวิทยาลัย	B.Eng.	วิศวกรรมเคมี	2538

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   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 โดยระบุสถานภาพในการทำการวิจัย ว่าเป็นผู้อำนวยการแผนงานวิจัย 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; (2006-2005
   - 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; (2009-2007
   - L(+)-lactic acid fermentation from cassava pulp by *Rhizopus oryzae*. (Thailand Research Fund – Seed Money; 480,000 Baht; (2009-2007
   - 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; (2010-2008

- Enhanced L(+)-lactic acid production by inhibition of alcohol dehydrogenase in *Rhizopus oryzae*. (TRF-MAG Window II Co-funding; 200,000 Baht; (2010-2008
- Screening of Thai native bacteria that give high lactic acid production. (Musashino Chemical Laboratory, Ltd., Japan; 1,985,000 Baht; (2010-2009
- Stopping hemorrhage by biodegradable copolymer wound dressing (prelim). (National Research University Project of CHE and Ratchadapiseksomphot Endowment Fund (AM007I); 320,000 Baht; (2011-2010
- 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; -20102011(
- 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(

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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 *terreus Aspergillus*บนเส้นใยธรรมชาติในถังปฏิกรณ์ชีวภาพแบบเบดสถิต ปีที่2

รายงานการรับ-จ่ายเงิน

	ประมาณการ	งบที่เกิดขึ้นจริง
รายได้		
เงินอุดหนุนงบประมาณแผ่นดิน	400,000.00	400,000.00
รวมรายได้	400,000.00	400,000.00
รายจ่าย		
หมวดค่าจ้างชั่วคราว	-	-
หมวดค่าตอบแทน	40,000.00	40,000.00
หมวดค่าใช้สอย	180,000.00	9,683.50
หมวดค่าวัสดุ	180,000.00	349,972.90
หมวดค่าครุภัณฑ์ (ถ้ามี)	-	-
รวมรายจ่าย	400,000.00	399,656.40
รายรับสูงกว่ารายจ่าย นำส่งส่วนการคลังจุฬาลงกรณ์มหาวิทยาลัย (เอกสารแนบ)		43.60
ดอกเบี้ย (ถ้ามี)		0.00
รวมจำนวนเงินที่นำส่งส่วนการคลังทั้งสิ้น		43.60

ขอรับรองว่ารายงานการรับ-จ่ายเงินข้างต้นเป็นความจริงทุกประการ

(ผู้ช่วยศาสตราจารย์ ดร. ณัฏฐา ทองจุล)

หัวหน้าโครงการวิจัย

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หมายเหตุ: รายงานตลอดโครงการเมื่อสิ้นสุดโครงการ/ปิดโครงการ