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Research

Optimization of bio-succinic fermentation process from crude glycerol by *Actinobacillus succinogenes*

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ABSTRACT

Bio-succinic acid is a commodity chemical with potent application in bioplastic and food industries which can be produced from renewable resources. In this study, bioprocess for production of bio-succinic from glycerol by *Actinobacillus succinogenes* was studied. The maximum succinic acid concentration in small-scale serum bottle experiments was 6.8 and 6.5 g/L using pure and crude glycerol, respectively, with supplemented $MgCO_3$. The ratio of acetic acid to succinic acid (AA/SA) implied the increased carbon flux to the C_4 pathway using crude glycerol supplemented with $MgCO_3$ compared to that with $CaCO_3$.The carbonate salts tended to induce C_3 metabolic pathway in fermentation using pure glycerol which was in accordance with the ratio of acetic acid to glycerol (AA/GL). The highest succinic acid concentration of 17.9 g/L from crude glycerol was achieved from batch fermentation in a lab-scale fermenter with the maximum glycerol utilization of 99.9% which were higher than those obtained from fed- batch and semi-continuous processes. Acetic acid tended to increase throughout the fermentation process in fed-batch and semi-continuous operations, which resulted in the lower product yield and substrate utilization efficiency. The time for initial purging of CO_2 showed effects on succinic acid production and internal metabolic pathways. This work provided a basis for process development on bio-succinic acid production from crude glycerol in industry.

Keywords: *Actinobacillus Succinogenes*, bio-succinic acid, carbonate salts, crude glycerol, fed-batch, semi-continuous

1. Introduction

Biodiesel is an interesting alternative fuel to replace fossil fuels because of its competitive energy properties and potential on production from agricultural products such as palm oil. According to the Alternative Energy Development Plan (AEDP) 2015-2036, reported that the Thai government plans to promote biodiesel production up to 14 million liters daily by 2036 [1]. In the biodiesel production process, 10 kg of crude glycerol (CG) with approximately 30-60% purity is generated from 100 kg of biodiesel produced. CG can be further purified to high purity glycerol with more than 90% purity for application in pharmaceutical, food and cosmetics industries. The rest of CG is discarded in landfills which can lead to environmental impacts. Some small biodiesel producers also burn excess CG as the way for its disposal which results in air pollution. Alternatively, CG can be used as a raw material for conversion to various products such as ethanol, animal feed, biogas, 1, 3-propanediol, polyunsaturated fatty acids, lipids, polyhydroxyalkanoates (PHA) and succinic acid through different biological processes [2-6].

Bio-succinic acid is a commodity chemical, which can be produced from renewable raw materials by environmentally-friendly microbial processes [5, 6]. Currently, most succinic acid is produced from maleic anhydride (MAN) by catalytic processes [7] which are energy intensive and generates chemical wastes. Succinic acid can be used for many applications, such as lubricant, solvent,

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and as ingredients in cosmetic and pharmaceutical products or can be converted to 1,-4 butanediol (BDO) and polybutyrate succinate (PBS) [8, 9]. Development of an efficient fermentation process for its production from inexpensive feedstock is thus of interest for industrial application.

Production of bio-succinic acid from diverse substrates has been reported using different microorganisms and mode of operations in fermentation. These microbes include *Mannheimia succinoproducens*, *Yarrowia lipolytica* and *Actinobacillus succinogenes* which resulted in the final succinic acid titer in the range of 9.4-151.4 g/L with the product yield ranging from 0.4-1.5 g/g [10-18]. Among various microbes, *A. succinogenes* is a potent succinic acid producer with advantages on its capability to produce high concentrations of succinic acid and high tolerance to acid produced during fermentation [19] However, studies on utilization of CG by *A. succinogenes* for succinic acid production have been limited to only very few reports [19] while its relevant metabolic pathways are not well characterized. In this work, we aimed to study the application of this bacterium for bio-succinic acid production from CG with the focuses on effects of carbonate salts and different operation modes including batch, fed batch and semi-continuous fermentation. The effects of CO_2 purging to the fermentation system were studied. The internal metabolic pathway was investigated based on the metabolites produced during fermentation to evaluate the efficiency on bio-succinic production. The work provides basis for valorization of CG to succinic acid for further development to industrial implementation.

2. Material and Methods

2.1. Materials

Crude glycerol *(CG)* and pure glycerol *(PG)* were obtained from Patum Vegetable Oil Co., Ltd, Pathum Thani, Thailand. Characteristics of CG and PG are summarized in Table 1.

2.2. Inoculum Preparation

A. succinogenes (ATCC 55618™) was purchased from ATCC® culture collection (www.atcc.org). Inoculum was re-cultivated from –20°C glycerol stock in 100 mL serum flasks, containing 20 mL of trypticase soy broth (TSB). After that, the serum bottle was purged with CO_2 (flow rate 1 L/min for 1 min) to create anaerobic conditions. Next, the serum bottle was incubated in a shaking incubator at 37°C with rotary shaking at 200 rpm for 24 h. After that, 10 mL of culture was added to the serum bottle containing 50 mL of TSB (20% v/v) and then purged with CO_2 (flow rate 1 L/min for 1 min). Finally, all serum bottles were incubated at 37°C with rotary shaking at 200 rpm for 12 h before use.

2.3. Effects of PG and CG and Carbonate Salts on Succinic Acid Production

The effects of using PG and CG with supplementation of different carbonate salts ($MgCO_3$ and $CaCO_3$) were studied in 100 ml serum bottles with a working volume of 50 mL. Each liter of the medium contained (in g/L): 5.0 g yeast extract, 8.4 g $NaHCO_3$, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgCl_2.6H_2O$, and 0.2 g $CaCl_2$ [5]. The initial concentration of glycerol in all conditions was fixed at 10 g/L. All conditions were inoculated with 20%v/v of inoculum and then purged with CO_2 at a flow rate of 1 L/min for 1 min. After that, the serum bottles were incubated in a shaking incubator at 37°C, 200 rpm. PG or CG were used as the main carbon source in bio-succinic acid fermentation. The concentration of carbonate salts ($MgCO_3$ and $CaCO_3$) was varied at 20, 40 and 60 mg/L to evaluate the effects of metabolic pathway on bio-succinic acid fermentation. All experimental data were performed in duplicate from two independent experiments. Mean values with standard deviation were reported.

2.4. Succinic acid Production in Continuous Stirred-Tank Reactor (CSTR)

The prepared free cells after re-cultivation were used for fermentation in a 1-L CSTR with a working volume of 720 mL. Each liter of medium contained (in g/L): 5.0 g yeast extract, 8.4 g $NaHCO_3$, 8.5 g $NaH_2PO_4.H_2O$, 15.5 g K_2HPO_4, 1.0 g $(NH_4)2SO_4$, 0.2 g $MgCl_2.6H_2O$ and 0.2 g $CaCl_2$ [5]. 120 mL of inoculum (20 % v/v) was added to the CSTR containing 600 mL of the medium supplemented with 1%w/v CG and 20 g/L $MgCO_3$. Before fermentation, the systems were initially purged with gaseous CO_2 at a flow rate 1 L/min for 1 min to provide anaerobic conditions. All experiments in the CSTR were controlled at 37°C with a mixing speed at 150 rpm. The experiments were designed to study the effect of operation modes including batch, fed-batch and semi-continuous operation. The amount of dissolved CO_2 was studied by varying the period of CO_2 purging for 1, 3 and 5 min to compare the efficiency of succinic acid production and the internal metabolic pathways by *A. succinogenes*. All operation modes were run in duplicate with two independent experiments. Mean values with standard deviation were reported.

2.4.1. Batch fermentation

This mode was started by mixing 120 mL of the initial inoculum (20% v/v) with 600 mL of the medium supplemented with 1%w/v of CG and 20 g/L $MgCO_3$. The mixture was fermented for 7 d (168 h) under the fixed condition at 37°C with mixing at 150 rpm.

2.4.2. Fed-batch fermentation

This mode was started by mixing 120 mL of the initial inoculum with 200 mL of the medium supplemented with 1%w/v of CG

Table 1. Characteristics of Glycerol

Glycerol types	Parameters			
	Purity (%wt.)	Water content (%wt.)	NaCl (%wt.)	Fatty acid and ester (%wt.)
CG	83.3	11.2	4.8	0.62
PG	99.9	0.047	-	0.2

and 20 g/L $MgCO_3$. The mixture was fermented for 24 h. After that, 100 mL of medium containing 1%w/v of CG supplemented with 20 g/L $MgCO_3$ was periodically added to the reactor every 24 h for 4 d. Finally, fermentation was further proceeded for 3 d without adding any medium to the reactor.

2.4.3. Semi-continuous fermentation

This mode was studied in two processes either with or without inoculum addition during CG fermentation in CSTR. Semi-continuous fermentation with inoculum addition was started similar to batch fermentation for 72 h. After that 240 mL of the fermented broth was drained while the new feed was added to the reactor every day to maintain a Hydraulic Retention Time (HRT) of 72 h. The mixture of the new feed was consisted of 120 mL of inoculum and 120 mL of culture medium with 1% w/v of CG and 20 g/L $MgCO_3$ while 240 mL of the culture medium with 1% w/v of CG and 20 g/L $MgCO_3$ was added to the fermentation mixture in the process without adding inoculum.

2.5. Analytical Method

Samples from all experiments were collected every 24 h to analyze the efficiency of succinic acid production. The samples were centrifuged at 10,000 rpm for 10 min. The supernatant was separated and analyzed using High Performance Liquid Chromatography (CTO-10AS VP, Shimadzu, Kyoto, Japan) equipped with a Refractive Index (RI) detector RID-10A and UV-VIS Detector SPD-20A/20AV, and an Aminex HPX-87H column (Biorad, Hercules, CA) to measure succinic acid and metabolites. The mobile phase comprised 5 mM H_2SO_4 solution at a flow rate of 0.5 mL/min and the column was controlled at 50°C.

3. Results and Discussion

3.1. Effect of carbonate salts on bio-succinic acid fermentation by *A. succinogenes* in serum bottle experiment

This section was designed to study the effects of carbonate salts on succinic acid production in term of metabolic pathway by *A. succinogenes* using PG and CG as the carbon source in small-scale experiments. Table 2 shows succinic acid concentrations, productivities, yields, the ratio of acetic acid to glycerol (AA/GL) and the ratio of acetic acid to succinic acid (AA/SA) from batch fermentation with different carbonate salts as an additive. According to the results, the maximum succinic acid concentration obtained were 6.8 and 6.5 g/L using PG supplemented with 40 g/L $MgCO_3$ and CG supplemented with 20 g/L $MgCO_3$, respectively. It was found that the metabolic pathway under these fermentation conditions supplemented with $MgCO_3$ was based on the C_4 pathway as suggested by the metabolite profiles. AA/SA ratio was decreased in both fermentation mixtures using either pure or crude glycerol with $MgCO_3$. In fermentation of CG with $CaCO_3$, AA/SA tended to increase during the fermentation process. Normally, glycerol degradation pathway by *A. succinogenes* is consisted of C_3 and

Table 2. Summary of Succinic Acid Concentration, Yield, Productivity, AA/GL and AA/SA from PG and CG Fermentation with Different Carbonate Salts in 0.1 L Serum Bottles at the 96th h Fermentation

Samples	Glycerol[a] (g/L)	Final [SA] (g/L)	Yield[b] (g/g)	Productivity[c] (g SA/L.h)	AA/GL[d] (g/g)	AA/SA[e] (g/g)	pH
1% PG							
no carbonate salts	10.6 ± (0.3)	2.5 ± (0.3)	1.1 ± (0.1)	0.5 ± (0.1)	0.03	0.14	6.68
20g/L $MgCO_3$	10.1 ± (0.0)	5.3 ± (0.3)	1.3 ± (0.1)	1.1 ± (0.1)	0.06	0.11	7.72
40g/L $MgCO_3$	10.2 ± (0.2)	6.8 ± (0.1)	1.1 ± (0.1)	1.4 ± (0.0)	0.05	0.07	7.88
60g/L $MgCO_3$	10.0 ± (0.4)	4.5 ± (0.4)	1.5 ± (0.1)	0.9 ± (0.1)	0.05	0.12	7.61
20g/L $CaCO_3$	10.2 ± (0.2)	3.7 ± (0.2)	1.1 ± (0.1)	0.8 ± (0.0)	0.05	0.13	6.96
40g/L $CaCO_3$	10.1 ± (0.2)	2.4 ± (0.2)	1.4 ± (0.2)	0.5 ± (0.0)	0.05	0.20	7.03
60g/L $CaCO_3$	10.4 ± (0.2)	3.3 ± (0.3)	1.2 ± (0.1)	0.7 ± (0.1)	0.05	0.15	6.92
1% CG							
no carbonate salts	9.7 ± (0.9)	2.6 ± (0.1)	3.9 ± (0.2)	0.6 ± (0.0)	0.05	0.19	6.85
20g/L $MgCO_3$	9. 9 ± (0.2)	6.5 ± (0.1)	2.1 ± (0.1)	1.3 ± (0.0)	0.04	0.06	7.41
40g/L $MgCO_3$	9. 9 ± (0.1)	6.1 ± (0.2)	1.1 ± (0.1)	1.3 ± (0.0)	0.04	0.06	7.48
60g/L $MgCO_3$	10.5 ± (0.3)	3.0 ± (0.1)	1.2 ± (0.1)	0.6 ± (0.0)	0.04	0.13	7.66
20g/L $CaCO_3$	10.3 ± (0.4)	1.1 ± (0.1)	1.6 ± (0.1)	0.2 ± (0.0)	0.01	0.11	7.17
40g/L $CaCO_3$	9.7 ± (0.2)	1.1 ± (0.1)	0.8 ± (0.1)	0.2 ± (0.0)	0.04	0.41	7.21
60g/L $CaCO_3$	10.1 ± (0.1)	0.9 ± (0.1)	0.5 ± (0.1)	0.2 ± (0.0)	0.04	0.45	7.11

Note: SA: succinic acid, PG: pure glycerol, CG: crude glycerol, AA: acetic acid, GL: glycerol
[a] Initial glycerol concentration
[b] Succinic acid yield on consumed glycerol
[c] Succinic acid volumetric production rate in g/L.h
[d] Ratio of produced acetic acid (AA) on consumed glycerol (GL)
[e] AA/SA ratio is g-AA/g-SA

C_4 pathways which involve generation of different main products. Succinic acid is the main final product of C_4 metabolic pathway whereas acetic acid, formic acid and ethanol are the main final products of C_3 metabolic pathway. Therefore, the ratio of AA/GL and AA/SA can suggest the internal metabolic process of the microbial cells that assimilate the dissolved CO_2 from $MgCO_3$ in dissociated form to drive succinic acid production by *A. succinogenes*.

The results showed that the reduction trend of AA/SA observed in CG fermentation was higher than that using PG in fermentation while it was increased in all cases of fermentation with $CaCO_3$ using either PG or CG. Previous studies revealed that $CaCO_3$ caused negative effect and resulted in the inhibition of bacterial cell growth, leading to a lower cell density in the fermentation mixture than that observed using $MgCO_3$. $MgCO_3$ is regarded as the most effective CO_2 supplier and buffer agent for bio-succinic production [20-21]. Besides, $MgCO_3$ can prevent flocculation and support dissolved CO_2 more than $CaCO_3$ [22-23]. However, the AA/GL ratio was increased in the fermentation using PG with carbonate salts compared with that without carbonate salts while this ratio was decreased for all cases using CG with carbonate salts. The results indicated that adding carbonate salts led to induction of the C_3 metabolic pathway in PG. This can be explained by the buffer capability of the carbonate salts. Compared to PG, CG contained a substantial amount of long chain fatty acids such as palmitic and oleic acid and 4.8% w/v NaCl. These impurities could inhibit anaerobic fermentation by the strain. Utilization of CG by the microbial cells may also be suppressed due to high concentrations of soluble chemicals and salts in the medium. This resulted in more complex metabolic scenarios on nutrient assimilation and limits utilization of CG by the pure strain. Under the suitable pH condition, PG can be assimilated more efficiently than CG and passed to the glycolysis pathway. Acetic and formic acid are then produced via C_3 pathway, and finally ATP is generated for driving microbial activities. Due to economic reason, CG was then used for the subsequent study on further optimization of the fermentation process.

3.2. Effect of Operation Modes in CSTR

The performance of *A. succinogenes* on fermentation of CG for succinic acid production was studied in 1-L CSTR fermenters. The processes were operated using different types of operation modes including batch, fed batch and semi-continuous fermentation. The process performance was compared based on the product yield and glycerol utilization efficiency.

3.2.1. Batch fermentation

In the batch operation mode, the results showed that succinic acid concentration increased continuously with the maximum succinic acid concentration of 17.9 g/L at the 96th h of fermentation. After that, it tended to decrease and stabilize until the end of batch fermentation (Fig. 1 (a)). Succinic acid concentration was correlated with glycerol concentration (Fig. 1 (b)). Glycerol concentration decreased continuously from the initial concentration of 10.2 g/L to the 96th h fermentation where the maximum glycerol utilization of 99.9% was reached, leading to the product yield of 1.8 g/g. The results indicated that the major metabolic pathway of CG fermentation by *A. succinogenes* was the C_4 pathway where succinic acid was the predominant type of end product while acetic and formic acid were minimally observed. The pH was decreased at the first 24st fermentation from the initial pH of 7.89 to the range of 7.13-7.77 throughout the fermentation.

3.2.2. Fed batch fermentation

In the fed-batch operation mode, the maximum succinic acid concentration was 10.4 g/L at the 96th h of fermentation (Fig. 2 (a)). After that, the succinic acid concentration was continuously decreased after the 120th h. This corresponded with the decrease of glycerol from an initial concentration of 9.6 g/L to 1.6 g/L at the 96th h of fermentation. The results indicated that *A. succinogenes* could use glycerol up to 99.7% w/w under this operation mode (Fig. 2 (b)). This led to the final product yield of 1.3 g/g. However, the trend of acetic acid produced during the fed batch fermentation was higher than that in the batch fermentation. This implied that the C_3 pathway showed an increasing role as the internal metabolic pathway of *A. succinogenes* for its growth under this condition. In addition, ATP generation via the C_3 pathway was necessary for this operation mode to support the internal activity of the bacterial cells. From the initial pH of 7.38, the pH was decreased to 6.85 at the 48st fermentation after adding the new medium into the reactor and then varying in the range of 7.10-7.93 throughout the fermentation.

3.2.3. Semi-continuous fermentation

The results showed that succinic acid concentration obtained from semi-continuous operation with addition of inoculum during fermentation was continuously increased. The maximum succinic

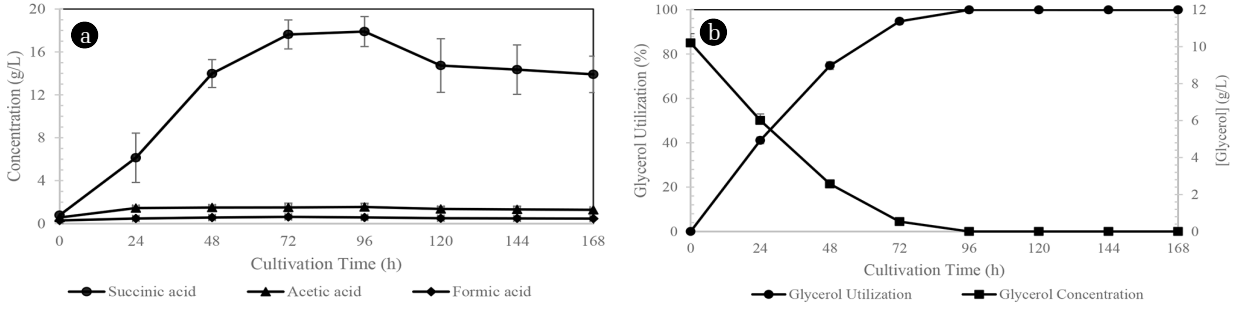

Fig. 1. Acid production and glycerol utilization during batch fermentation in 1-L CSTR. The process was operated using 1% CG and 20 g/L $MgCO_3$for7d under the fixed condition at 37°C with mixing at 150 rpm (a) Succinic (●), acetic (▲) and formic (◆) acid concentration; (b) glycerol utilization (●) and glycerol concentration (■).

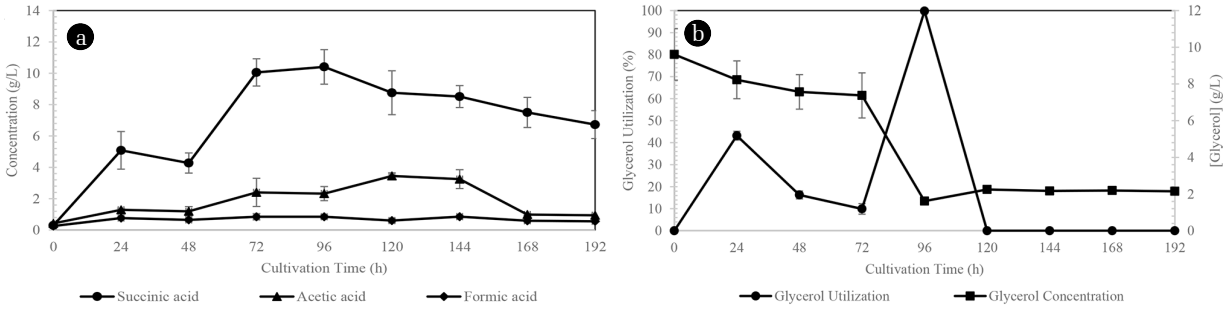

Fig. 2. Acid production and glycerol utilization during fed-batch fermentation in 1-L CSTR. The new medium containing 1% CG and 20 g/L $MgCO_3$wasperiodicallyaddedtothereactorevery24hoursfor4 days and further proceeded for 3 d without adding any medium to the reactor (a) Succinic (●), acetic (▲) and formic (◆) acid concentration; (b) %glycerol utilization (●) and glycerol concentration (■).

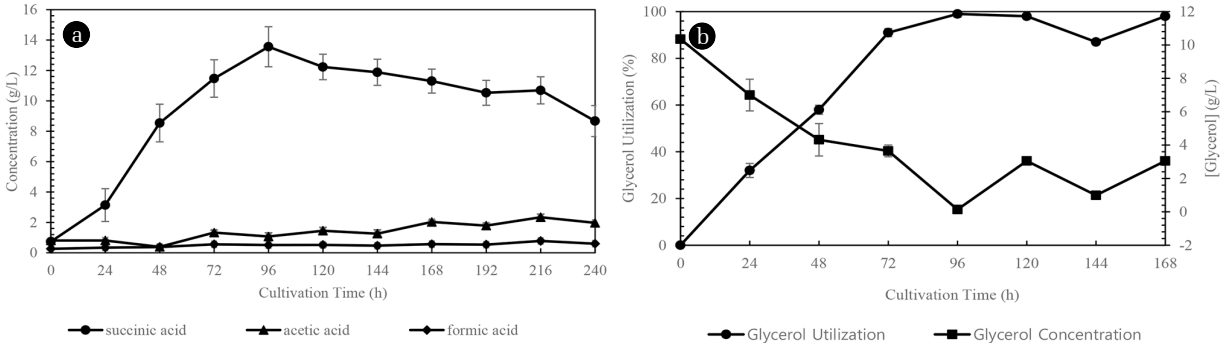

Fig. 3. Acid production and glycerol utilization during semi-continuous fermentation with inoculum addition in 1-L CSTR by keeping the HRTs of 3 days with 1:1 mixture of the medium containing 1%CG and 20 g/L $MgCO_3$andtheinoculum.(a)Succinic(●), acetic (▲) and formic (◆) acid concentration; (b) %glycerol utilization (●) and glycerol concentration (■).

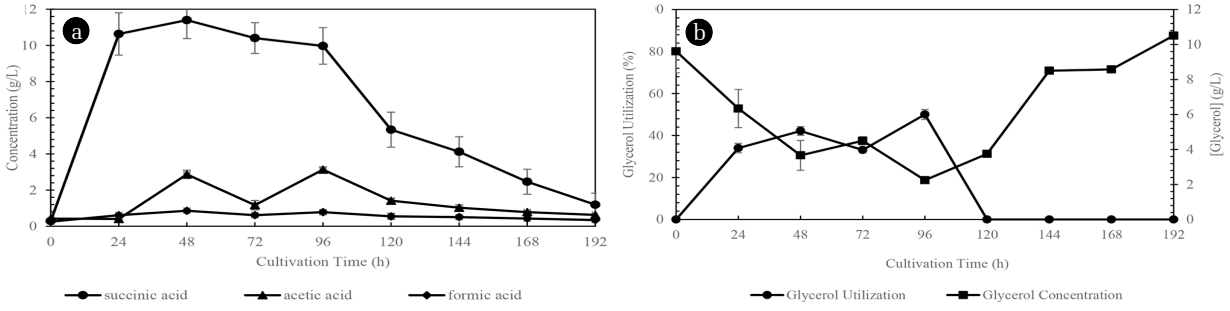

Fig. 4. Acid production and glycerol utilization during semi-continuous fermentation without inoculum addition in 1-L CSTR by keeping the HRTs of 3 days with addition of 1%CG and 20 g/L $MgCO_3$ with noinoculum addition) (a) Succinic (●), acetic (▲) and formic (◆) acid concentration; (b) glycerol utilization (●) and glycerol concentration (■).

acid concentration was 13.6 g/L observed at the 96th h of fermentation. In contrast, acetic and formic acids were observed at low concentrations that remained in the range of 0. 7 - 2.3 g/L and 0.3 – 0. 8 g/L, respectively (Fig. 3 (a)). Glycerol utilization reached 96.3% w/w at the 96th h (Fig. 3 (b)), leading to the maximum product yield of 1.3 g/g. After adding the new feed, glycerol utilization was maintained in the range of 87.0-99.0% w/w until the end of the experiment (240th h; data not shown). The pH showed a decreasing trend from 7.67 to 6.50 and lower than those from batch and fed batch operations.

Fig. 4 (a) and (b) showed results of CG fermentation in the semi-continuous operation without addition of inoculum during fermentation. The maximum succinic acid was 11.4 g/L at the first 48th h of fermentation, equivalent to the product yield of 1.9 g/g. After that, succinic acid concentration was continuously decreased. Acetic and formic acid concentrations were 0.4 – 3.1 g/L, and 0.3 – 0.9 g/L, respectively (Fig. 4 (a)). The maximum glycerol utilization under this condition was 50.0% at the 96th h of fermentation while no further glycerol consumption was observed after this time point. Glycerol was gradually accumulated from the new feeding. The pH in the system tended to increase after adding the new medium and drainage the fermented broth that was in the range of 7.08-9.31. Some of the bacterial cells were lost during draining of the cultured broth from the CSTR every day to maintain

the HRTs of 3 d. This resulted in the lower amount of cells remained in the reactor which affected glycerol assimilation and led to accumulation of glycerol in the mixture during the 120^{th} -192^{th} h. Moreover, the bacterial cells could not be promptly proliferated and grown. Addition of the bacteria into the system during the fermentation process was thus found to directly affect the overall efficiency of the fermentation system in terms of both glycerol assimilation and succinic acid production.

3.3. Effect of Dissolved CO_2 from Initial CO_2 Purging on Batch Fermentation in CSTR

In this part, the effects of CO_2 purging period on succinic acid production via the C_4 pathway by *A. succinogenes* was studied by adding gaseous CO_2 during batch fermentation. Co-fermentation of CO_2 was reported for succinic acid production by *A. succinogenes*. Fermentation with a higher concentration of dissolved CO_2 was reported to increase the ratio of succinic acid to the other metabolites, the ratio of carbon recovery, and finally the succinic acid yield [24]. According to our results, it is indicated that not only $MgCO_3$ but also the period of initial CO_2 purging to start up the fermentation led to different performance of the bacteria on both glycerol uptake and internal metabolic process via the C_3 and C_4 pathways. Fermentation with initial CO_2 purging for 1 min resulted in a higher concentration of succinic acid than fermentation with initial CO_2 purging for 3 and 5 min (Fig. 5 (a)). The results illustrated that the highest succinic acid concentration of 17.9 g/L was achieved at the 96^{th} h of fermentation and then decreased until the end of batch fermentation. Similarly, the trend of glycerol utilization under the condition with initial CO_2 purging for 1 min was higher since the start of the batch process (Fig. 5 (b)). The maximum glycerol utilization reached 94.8% within 72 h, while glycerol utilization under the condition of purging CO_2 for 3 and 5 min was slower (Fig. 5 (b)). Regarding acetic and formic acid production, metabolites in the C_3 pathway were higher throughout the fermentation process under the condition of purging CO_2 for 3 and 5 min compared with the condition of purging CO_2 for 1 min (Fig. 5 (c) and (d)). The results suggested that gaseous CO_2 could be used as both co-substrates to support the C_4 pathway through the reverse TCA cycle and as a main factor in growth and proliferation of the cells via the C_3 pathway. In general, the C_3 pathway induced not only acetic and formic acid production, but also generation of ATP to use as an energy source of the microbial cells for their internal activities. The extended purging of gaseous CO_2 could result in increasing dissolved CO_2 along with a high partial pressure of CO_2 in the fermentation system. This result agreed with the related research of Xi et al. (2011) [25] which investigated the effect of different CO_2 partial pressure on CO_2 fixation by *A. succinogenes*. They reported that the condition with 0.1 MPa of CO_2 resulted in the maximum amount of dissolved CO_2 in the broth. The maximum amount of dissolved CO_2 (dCO_2) of 22.7 mM and dry cell weight were obtained. Additionally, gaseous CO_2 can enter the cell membrane and be used as a substrate for the microbial cells. On the other hand, HCO_3^- and CO_3^{2-} need ATP in the process to bind with protein and transport to bacterial cells [26]. Normally, gaseous CO_2 can be used by the microbial cells under the conditions containing HCO_3^-, CO_3^{2-} and gaseous CO_2 [27]. Therefore, dCO_2

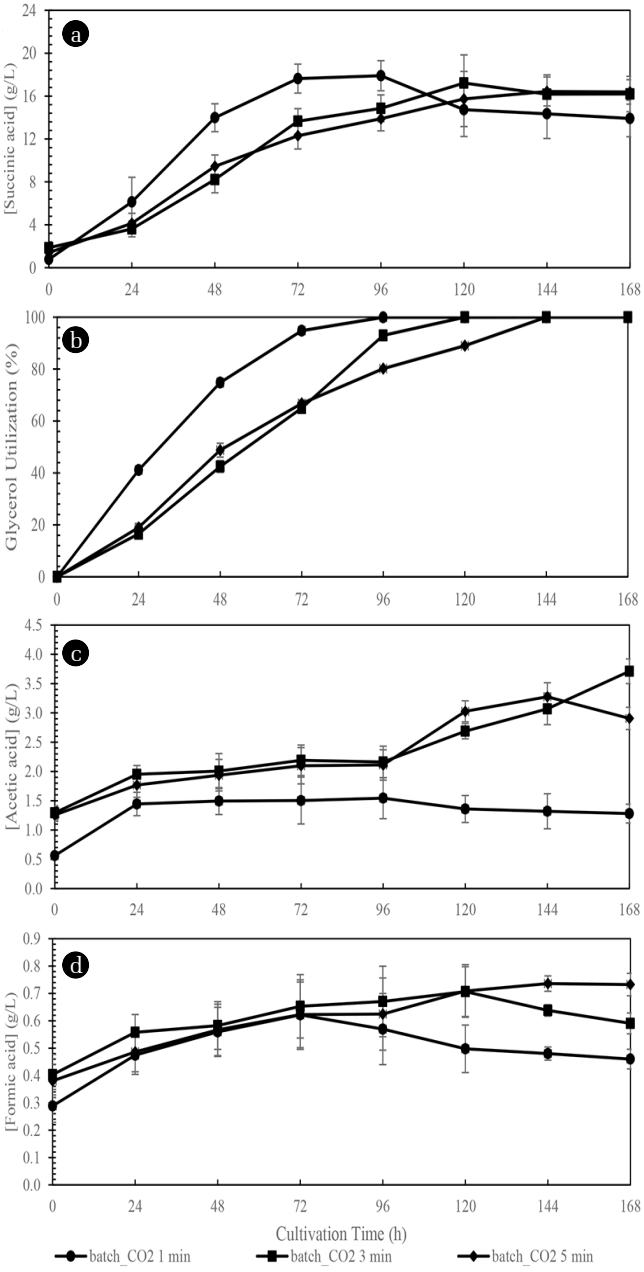

Fig. 5. Effect of CO_2 purging on succinic acid production from CG in batch process. (a) Succinic acetic concentration, (b) Glycerol utilization, (c) Acetic acid concentration and (d) Formic acid concentration The symbols of ●, ■ and ◆ represent batch fermentation by purging the initial CO_2 for 1, 3 and 5 min, respectively.

in the broth was directly related to CO_2 availability inside the bacterial cells. This is controlled to maintain the PCO_2/H_0 ratio according Henry's Law [28]. Moreover, HCO_3^- and CO_3^{2-} could dissociate from the CO_2 balance and were converted to dissolved CO_2 when the rate of CO_2 utilization was higher than the rate of CO_2 degradation in biomass fermentation. Therefore, it is possible that the HCO_3^- produced during the fermentation process by purging

Table 3. Comparison of Succinic Acid Production by *A. Succinogenes* and Other Microbes Using Different Substrates

Feedstock	Fermentation conditions	Yield (g SA/g substrate)	Final [SA] (g/L)	References
Carob Pods	Batch fermentation with 35 g/L sugar, 0.05 vvm CO2, 37˚C	0.54	9.4	[10]
	Fed batch maintaining a constant sugar concentration around 15 g/L in abioreactor	0.94	18.6	
Oil palm trunk sap	Batch fermentation in bioreactor with oil palm trunk as carbon source.	0.52	13.6	[11]
Fresh cassava root	Fed batch, 70 g/L glucose, 40%wt $MgCO_3$, 35˚C	1.51	151.4	[12]
Duckweed	Batch fermentation in 1.3 L bioreactor with corn steep liquor powder as nitrogen source	0.90	57.8	[13]
	Semi-simultaneous saccharification and fermentation	0.80	72.7	[14]
Bagasse	Batch fermentation, 15 g/L glucose, 0.3vvm CO_2, 37˚C	0.45	33.6	[15]
Glucose	Batch fermentation, 10 g/L $MgCO_3$ by *Mannheimia succinoproducens*	0.55	9.8	[16]
Glycerol	Batch fermentation with combined nitrogen sources (Corn Steep Liquor: tryptone at the ratio of 2:1 (w/w)) by *Yarrowia lipolytica*	0.41	11.5	[17]
Glycerol	Batch fermentation, 10 g/L glycerol, 0.04vvm CO_2, 37˚C by *A. succinogenes*	0.85	12. 8	[18]
Crude glycerol	Batch fermentation in 1L bioreactor, 1 min CO_2 purge, 20 g/L $MgCO_3$	1.75	17.9	This study

the initial CO_2 for 3 and 5 min and CO_3^{2-} dissociated from $MgCO_3$ could pass into the bacterial cells using ATP in the initial period. The internal reaction of metabolite flux to the C_3 pathway resulted in production ATP, acetic and formic acid at the 24^{th}-96^{th} h of fermentation. This was evidently observed at the 120^{th}-168^{th} h of fermentation under the condition of CO_2 purging for 3 and 5 min which was also higher than the condition with purging CO_2 for 1 min. The amount of HCO_3^- and CO_3^{2-} permeated into the microbial cells and converted to dissolved CO_2 could promote succinic acid production via the C_4 pathway. This corresponded to glycerol utilization remained in the 96-168^{th} h fermentation in both cases. In summary, the period of initial CO_2 purging in the fermentation system affected glycerol utilization, HCO_3^- and CO_3^{2-} absorption, and finally the internal metabolic pathway related to succinic production by *A. succinogenes*.

The succinic acid concentration and yield obtained in our work based on the batch fermentation in CSTR using CG as the carbon source are in the same range to those reported in previous works (Table 3). *A. succinogenes* was studied for succinic acid production from different renewable carbon sources, for examples, glucose, oil palm trunk sap, carob pods, bagasse, duckweed and fresh cassava roots which resulted in the final succinic acid concentration and product yield of 9.4-151.4 g/L and 0.4-1.5 g/g., respectively [10-18]. The product titer and yield obtained in our study were also comparable to those previously reported using *A. succinogenes* with glycerol as the substrate in batch fermentation which was 12.8 g/L and 0.9 g/g [18]. Considering the current cost of CG from local biodiesel producer (0.12-0.15 USD/L) [29] and the price of succinic acid (0.9 USD/kg) [30], there is still a margin for the developing technology for economically feasible production of succinic acid from CG. The feasibility can be further improved by enhancing performance of the microbial strains by genetic engineering techniques together with the development and optimization of bio-processes with cost improved effectiveness.

4. Conclusions

A bioprocess for production of bio-succinic acid from CG by *A. succinogenes* was reported in this study. The batch process was found to be more efficient compared to the fed-batch and semi-continuous operations under the experimental conditions according to the product concentration and glycerol utilization. Dissolved CO_2 and carbonate salts were important factors on driving the internal metabolic pathway to bio-succinic production through the C_4 pathways. The flux to the C_4 pathway was more obviously observed using CG compared to PG fermentation together with the preference of $MgCO_3$ over $CaCO_3$. The work provided a basis for process development on bio-succinic acid production from CG in industry.

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Author Contributions

S.K. (Assistant Professor) designed the study, conducted the experiments, analysed and discussed the results and wrote the manuscript. V.C. (Ph.D.) supervised the project and proved the manuscript. C.S.

(Assistant Professor) discussed the results and contributed to the final manuscript. N.P. (Assistant Professor) proved the manuscript.

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