Title:

High Production Yield and Specific Productivity of Succinate from Cassava Starch by Metabolically-engineered *Escherichia coli* KJ122

Running title:

High yield and productivity of succinate from cassava starch by *E. coli* KJ122

Authors:

Kirin Khor¹, Apichai Sawisit¹, Sitha Chan¹, Sunthorn Kanchanatawee¹, Sirima Suvarnakuta Jantama², Kaemwich Jantama^{1,*}

Affiliated Addresses:

¹ Metabolic Engineering Research Unit, School of Biotechnology,
Institute of Agricultural Technology, Suranaree University of Technology, 111 University
Avenue, Suranaree sub-district, Muang district, Nakhon Ratchasima, Thailand, 30000

² Division of Biopharmacy, Faculty of Pharmaceutical Sciences, Ubon Ratchathani
University, Warinchamrap, Ubon Ratchathani, Thailand, 34190

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***Corresponding author:**

Professor Kaemwich Jantama, PhD
Metabolic Engineering Research Unit, School of Biotechnology,

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Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Suranaree sub-district, Muang district, Nakhon Ratchasima, Thailand, 30000
Phone: +66-66-44-224-562; Fax: +66-44-224-154; E-mail: kaemwich@sut.ac.th

ABSTRACT

BACKGROUND: Succinate has been widely used in chemical industries and its microbial production is a desirable option. The feasibility of bio-based succinate production on an industrial scale strongly depends on the utilization of cheaper renewable resources. The use of cassava starch may make the fermentation process of succinate more economically competitive.

RESULTS: Simultaneous saccharification and fermentation (SSF) of succinate production was performed in minimal salt medium containing a cheap cassava starch. The parameters affecting succinate production from cassava starch by *E. coli* KJ122 were optimized. A succinate concentration of $70.1 \pm 0.1 \text{ g l}^{-1}$ was produced with a yield of $1.00 \pm 0.01 \text{ g g}^{-1}$ substrate and productivity of $0.97 \pm 0.01 \text{ g l}^{-1} \text{ h}^{-1}$ during batch SSF in a 2l fermentor under optimized conditions. Further improvements in succinate concentration ($82.5 \pm 0.7 \text{ g l}^{-1}$), yield ($1.03 \pm 0.01 \text{ g g}^{-1}$ glucose), and average and specific productivities of $1.15 \pm 0.01 \text{ g l}^{-1} \text{ h}^{-1}$, and $456 \text{ mg g CDW}^{-1} \text{ h}^{-1}$ respectively were observed in fed-batch SSF.

CONCLUSION: *E. coli* KJ122 produced a succinate yield of 92% of theoretical maximum and the highest specific productivity ever reported. Succinate production yield and specific productivity from cassava starch in this study are superior to other previously published works. *E. coli* KJ122 may be used as a biocatalyst for cost-effective succinate production.

Keywords: Succinate; Cassava starch; *E. coli*; Simultaneous saccharification and fermentation; Metabolic engineering

INTRODUCTION

Succinic acid has been used widely in agricultural, food, green solvent, and pharmaceutical industries. Currently, it is valued as one of the key platform chemicals used directly in the preparation of biodegradable polymers, including polybutylene succinate (PBS) and polyamides. It is also used as a raw material to synthesize fine chemicals of the C4 family.¹ In recent years, the biological production of succinic acid from renewable resources has attracted significant interest because its production by fermentation has advantages over chemical processes.² Additionally, succinic acid production by fermentation has distinct advantages over the production of other organic acids due to the consumption of CO₂ thus reducing greenhouse gas.³ These factors provide an incentive for microbial succinate production.

Investigations of succinic acid production were carried out with many rumen bacteria such as *Actinobacillus succinogenes* and *Anaerospirillum succiniproducens*.⁴⁻⁵ However these microorganisms require more special and complex nutrients, including yeast extracts, peptone, and vitamins, thus increasing costs involving medium, purification, separation, and waste disposal. Unlike rumen bacteria, a metabolically-engineered *Escherichia coli* strain produces succinic acid under simple anaerobic conditions in minimal salt medium containing

solely glucose without antibiotics for maintenance and expression of heterologous genes.⁶ However, the feasibility of bio-based succinic acid production on an industrial scale strongly depends on the utilization of cheaper renewable resources to minimize the cost of the fermentation process.⁷ Therefore, use of an inexpensive carbon source, such as cassava starch, may provide the fermentation process that produces succinic acid that is more economically competitive by *E. coli* strains than by other rumen bacteria.

Cassava is cheap and readily available in tropical and sub-tropical areas, especially Thailand. Moreover, cassava is believed to be the cheapest source of starch compared to cereals, tubers, and root crops.⁸ Cassava usually contains 70-75% (w w⁻¹) of starch, which is glucose polymers linked with $\pm$ -1,4- and $\pm$ -1,6-glycosidic bonds. Starch can be directly converted to fermentable sugar via an enzymatic hydrolysis by a combination of glucoamylase and $\pm$ -amylase without performing any pre-treatment processes. Even though many studies considered the utilization of cassava starch as a substrate for fermentation, reports did not show the production of succinic acid from cassava starch. Some of these works produced ethanol, lactic acid, or other fermentative products.⁹⁻¹² Only one study focused on succinate production from cassava starch by *E. coli* NZN111.¹³ However, a succinate yield of only 0.7 g g⁻¹ cassava starch, and the specific productivity of 338 mg g⁻¹ CDW h⁻¹ were obtained in a complex medium. Also, the fermentation process had to generate high biomass prior to producing succinate thus increasing the costs of medium and carbon substrates. The succinate production procedure, yield, and productivities obtained were still far from being economically competitive.¹

Recently, *E. coli* KJ122 was developed by a combined strategy of metabolic engineering and metabolic evolution to produce succinate under anaerobic conditions. The *E.*

E. coli KJ122 produced a high succinate yield at 95% of the theoretical maximum (1.62 mol/mol glucose utilized).⁶ *E. coli* strain KJ122 may be useful as a biocatalyst for the commercial production of succinate using starchy materials as carbon sources. Therefore, this study investigated and optimized parameters affecting succinate production from cassava starch. The fermentations were carried out in mineral salt medium without supplementation of any rich or expensive nutrients by metabolically engineered *E. coli* KJ122 under simple anaerobic batch fermentation. To the best of the authors' knowledge, this work originally revealed the highest yield (1.03 g g⁻¹ cassava starch) and the highest specific productivity (456 mg g⁻¹ CDW h⁻¹) compared to previously-published works (Table 1) on the use of cassava-derived substrates for succinate production by metabolically engineered *E. coli*.

EXPERIMENT

Cassava starch and enzyme

Cassava starch was purchased from Korat Starch Industry (Nakhon Ratchasima, Thailand). The cassava starch was stored in a desiccator until used. Its moisture content was 9.65±0.33% (w w⁻¹). The commercial enzymes Glucoamylase (AMG) and α -amylase (Amy) were purchased from Siam Victory Chemicals Co., Ltd., Bangkok, Thailand, with activities of 213,000 U ml⁻¹ and 42,169 U g⁻¹ respectively.

Growth condition of strains and media

E. coli KJ122 was kindly provided by the Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA. A new low salt medium, AM1¹⁴ (4.2 g l⁻¹ total salts) was used in the fermentation with the KJ122 strain. The medium was sterilized by autoclave for 15 min. Seed cultures and fermentation were grown at 37°C; 200 rpm in

AM1 mineral salt mediums containing 2% (w v⁻¹) glucose, 100 mM KHCO₃, and 1 mM betaine at an initial pH of 7.0. Subsequently, pH was maintained during fermentation by the automatic addition of a mixture solution of 3 M KOH and 1.5 M K₂CO₃. A mixture solution was prepared by adding 3 M KOH and 1.5 M K₂CO₃ at the mixing volumetric ratio of 6:1. Fermentations were inoculated at the cell concentration of 0.1 g l⁻¹, and carried out in small fermentation vessels with a working volume of 350 ml l⁻¹. Anaerobiosis was rapidly achieved during the growth phase by bicarbonate served to ensure an atmosphere of CO₂.

Optimization of succinic acid production from cassava starch by simultaneous saccharification and fermentation (SSF)

To study the effect of pH on succinic acid production, the fermentation was carried out with the initial pH adjusted to 6.0, 6.5, and 7.0, and maintained using base additions throughout the fermentation. Also, the effect of temperature on succinic acid production by SSF process was studied by carrying out fermentation at temperatures ranging from 37°C to 43°C. To determine the effect of substrate concentrations, different concentrations of cassava starch ranging from 70-150 g l⁻¹ were tested. Additionally, optimal enzyme loading of AMG was also evaluated by varying the concentrations of AMG in the range of 300-1,300 U g⁻¹ cassava starch. An initial cell biomass of KJ122 strain was provided to the fermentation at the optical density at 550 nm of 0.6. The incubation time was prolonged to 72 h. All experiments were performed at least three times.

Succinic acid production by simultaneous saccharification and fermentation (SSF) in a 2L fermentor

In a batch process, cassava starch (70 g l⁻¹) and 100 U Amy g⁻¹ cassava starch were added to de-ionized water before autoclave for a liquefaction step. The fermentation was

initiated by inoculating a seed culture, and carried out at 37°C at an initial pH of 7.0. The pH was controlled by the automatic addition of mixture of 3 M KOH and 1.5 M K₂CO₃ when the pH was lower than 6.5. In fed-batch SSF, cassava starch at concentration of 50 g l⁻¹ was initially fed to fermenter. The feeding pattern was done by the intermittent strategy. Liquefied cassava starch solution was added simultaneously with AMG when the residual glucose concentration in the fermentation broth was reduced to 15 g l⁻¹. The experiments were repeated at least two times. Samples were removed from the fermentor every 8 h till 72 h for analysis.

Analytical method

Three milliliters of samples were removed during fermentation every 8 h and centrifuged at 10,000 g for 5 min. The supernatant and cells were separated and used for further analysis. The moisture content of the cassava starch was estimated by a drying machine at 130°C. The reducing sugars presenting in the fermentation broth were measured using DNSA method.¹⁵ At incubation time zero till 16 h, cell concentrations from fermentation broth were determined by using a modified method of Thang and colleagues.¹⁶ From 24 h to 72 h, the cell biomass was estimated from the optimal density at 550 nm (1 OD₅₅₀ = 0.33 g CDW l⁻¹). Organic acids and sugars were determined by the use of high performance liquid chromatography, HPLC, (Agilent, 2009) equipped with UV and refractive detectors with a Bio-Red Aminex HPX-87H ion exclusion column. The supernatant was filtered through a 0.2 µm filter membrane before injection into HPLC. The succinate production yield was calculated based on the potential glucose in the medium with the assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose.¹⁷

Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL). Triplicate determinations were performed on each test and averages were used in the report. The differences among mean values were established using Duncan's multiple-ranges test (DMRT) at a 95% significance level.

RESULTS

Effects of significant parameters on succinate production during the SSF process by *E. coli* KJ122

Effects of cassava starch concentrations

At a high substrate concentration, uncompetitive-substrate inhibition **is usually observed**, thus deactivating enzymatic reactions and inhibiting microbial growth during the SSF process.¹⁸ To achieve a suitable level of cassava starch used during SSF, the effects of cassava starch concentrations ranging from 70 to 150 g l⁻¹ on succinate production by *E. coli* KJ122 were investigated. The results showed that the highest succinate concentration of 66.0±2.8 g l⁻¹ was obtained with the yield of 0.46±0.02 g g⁻¹ cassava starch utilized when cassava starch was provided at the concentration of 150 g l⁻¹ (Figure 1). However, the succinate concentration was not significantly different when compared with that achieved from 70, 110, or 130 g l⁻¹ of cassava starch. The highest succinate production yield of 0.91±0.03 g g⁻¹ cassava starch was obtained when 70 g l⁻¹ of cassava starch was used. It was likely that the succinate yield dramatically decreased when the cassava starch concentration was increased in the fermentation broth. In addition, glucose still remained in the fermentation broth in all conditions, even when the incubation time was prolonged to 72 h. The remaining glucose

concentration significantly accumulated when more cassava starch was provided to the fermentation broth (Figure 1). This result thus reflected lowered succinate yields at increased levels of cassava starch concentrations. In addition, biomass concentration was still highest when 70 g l⁻¹ cassava starch was provided, and dramatically decreased when concentrations of cassava starch greater than 90 g l⁻¹ were supplied.

Effects of pH

The enzymatic activity of AMG for saccharification of starchy materials is optimal at pH ranging from 3.5 and 5.5 according to the manufacturer's specification. However, the cultivation of *E. coli* KJ122 at pH less than 5.5 during the SSF process is impossible. Thus the effects of the pH of the medium on the growth and succinic acid production of *E. coli* KJ122 were also considered in this study. The results showed that an improvement of growth and biomass was observed when the pH was increased from 5.5 to 7.0. As expected, the succinate production was significantly enhanced when pH was increased from 5.5 to 6.5. The highest succinate concentration of 63.5±0.2 g l⁻¹ was obtained at pH 6.5 that was considered as the same level as that obtained at pH 7.0 (62.8±2.3 g l⁻¹). In addition, the succinate production yield of 0.91 g g⁻¹ cassava starch utilized was observed at pH values of 6.5 and 7.0 while yields at pH 5.5 (0.37±0.02 g g⁻¹) and pH 6.0 (0.72±0.01 g g⁻¹) were significantly lower. Glucose also remained at the least concentration of 0.85±0.03 g l⁻¹ when the strain KJ122 was cultivated at pH 7.0 compared to those at lower pH values (Figure 2). The results may suggest that decreases in growth and biomass production at pH values less than 6.0 resulted from a sufficient high acid concentration collapsing the pH gradient across the cell membrane.

Effects of AMG loading

The price of AMG used during SSF process may still be problematic for succinic acid production on a large scale. Nevertheless, the cost can be significantly reduced if the amount of enzyme loaded during the SSF process decreases. Liquefied cassava starch (70 g l^{-1}) was saccharified by adding AMG at concentrations ranging from 300-1300 U/g cassava starch during the SSF process at pH 6.5. At 300U AMG g^{-1} cassava starch, only $55.6 \pm 0.3 \text{ g l}^{-1}$ of succinic acid was achieved with the yield of $0.79 \pm 0.01 \text{ g g}^{-1}$ cassava starch. However, a further increase in AMG loading in this range of 500 to 1,300 U AMG g^{-1} cassava starch affected succinic acid production in terms of both concentration and yield. Succinate concentrations produced greatly improved and ranged from 62-64 g l^{-1} with yields of 0.89-0.91 g g^{-1} cassava starch. The use of enzyme in the range of 300-500 U AMG g^{-1} cassava starch provided a negative effect on the remaining glucose concentrations compared to those of enzyme loadings greater than 500 U AMG g^{-1} cassava starch (Figure 3). It was presumed that the lower AMG loading affected the saccharification efficiency of starch in which a degree of polymerization of glucose in the liquefied cassava starch might be higher than that of conditions that contained higher enzymatic loadings (700-1,300 U AMG g^{-1} cassava starch). The strain KJ122 may delay or retard consumption of a higher degree of polymerized glucose chains. This led to higher amounts of glucose remaining in the fermentation broth. Surprisingly, the use of enzyme loading at any levels did not adversely affect the growth of the strain KJ122 (Figure 3).

Effects of temperature

The efficiencies of bioprocesses are strongly temperature-dependent due to the dependence of enzymatic activity and cellular maintenance upon temperature. The optimum temperature of AMG enzyme that permits total hydrolysis of starch to fermentable glucose is between 58

and 60°C as per the manufacturer's recommendation. However, *E. coli* KJ122 has an optimal temperature for growth in the range of 37 to 43°C.¹⁹ The growth of *E. coli* KJ122 usually stalls if the temperature exceeds 45°C.⁶ In this experiment, it was essential to compromise two optimal temperatures for both bacterial growth and saccharification by AMG during the SSF process at temperatures ranging from 37 to 43°C. The results showed that the most favorable temperature for succinate production, cell growth, and glucose utilization by the strain KJ122 was 41°C. The highest succinate concentration of $66.9 \pm 5.1 \text{ g l}^{-1}$ with the yield $0.96 \pm 0.03 \text{ g g}^{-1}$ cassava starch was observed at 41°C. Nevertheless, temperatures ranging from 37-41°C seemed to exert little effect on succinate concentration and yield. Glucose concentration at 37°C was higher than those between 39 and 41°C. At 43°C, growth ($0.27 \text{ g CDW l}^{-1}$), succinate concentration ($3.1 \pm 0.2 \text{ g l}^{-1}$), and yield (0.04 g g^{-1} cassava starch) were lowest. This suggested that a temperature higher than 41°C was not suitable for growth thus resulting in less substrate consumption and very low succinate production by *E. coli* KJ122 strain (Figure 4).

Succinate production in batch and fed-batch SSF in a 2l fermenter

Batch SSF experiments were performed in a 2l bioreactor with a working volume of 1.5 l. The optimized parameters obtained from the previous experiments were used for succinate production as follows: 70 g l^{-1} cassava starch, 500 U AMG g^{-1} cassava starch, pH 6.5 at 39°C at 200 rpm for 72 h. Figure 5a represents succinate production using liquefied cassava starch as the carbon source under anaerobic conditions during batch SSF by the *E. coli* KJ122 strain. Liberated glucose was a main product from cassava starch hydrolyzed after adding AMG into the bioreactor. The maximum concentration of the glucose was at 8 h incubation time. After

that, it started to decrease and was completely consumed by the strain KJ122 at 72 h. The maximum succinic acid production was reached at $70.1 \pm 0.1 \text{ g l}^{-1}$ at 72 h with the average productivity of $0.97 \pm 0.01 \text{ g l}^{-1} \text{ h}^{-1}$.

The SSF process should be performed at high substrate concentrations to lower the overall demand of process energy and water consumption, thus decreasing the production cost.²⁰ However, batch SSF processes are difficult to perform with high dry matter content due to the high viscosity of the medium. Fed-batch is the preferred production strategy when high nutrient concentrations affect the productivity and the yield. To increase higher succinate production efficacy, fed-batch SSF was conducted in this study. In fed-batch SSF, the experiments were started with the optimized parameters (pH, AMG concentration and temperature) as same as performed in a batch SSF. AMG was added in portions simultaneously with the liquefied cassava starch. The liquefied solution of cassava starch (300 g l^{-1}) was added to the bioreactor to keep an appropriate glucose concentration at 30 g l^{-1} . Figure 5b represents the succinate production after two supplementations of liquefied cassava starch at 20 h and 28 h. The maximum concentration of succinate was enhanced to $82.5 \pm 0.7 \text{ g l}^{-1}$ with the yield of $1.03 \pm 0.01 \text{ g g}^{-1}$ and productivity of $1.15 \pm 0.01 \text{ g l}^{-1} \text{ h}^{-1}$. Also, the specific productivity of $456 \text{ mg g CDW}^{-1} \text{ h}^{-1}$ was observed. In this present study, the yield of succinate production was equivalent to $1.66 \text{ mol mol}^{-1}$ glucose used. The results confirmed that succinate concentration, yield, and productivity could be improved by the fed-batch process in comparison to the batch SSF process.

DISCUSSION

In a bio-process, the cost of medium is one of the key factors determining the feasibility of the fermentative production of bulk bio-chemicals including succinic acid. Therefore, in this study, cassava starch as a low cost agricultural product was evaluated for succinate production by a metabolically-engineered *E. coli* KJ122 strain. In addition, parameters affecting succinate production efficiency from cassava starch under the SSF process by the strain KJ122 were optimized. Cassava concentration was optimized by comparison of succinate concentration, residual glucose, and yield. It is noted that succinate concentrations increased when initial cassava starch concentrations were increased. But the production yield dramatically decreased when initial cassava starch concentrations from 70 to 150 g l⁻¹ were increasingly provided. However, titers of succinate from 110 to 150 g l⁻¹ cassava starch were not significantly different compared to that obtained from 70 g l⁻¹ cassava starch. In addition, increasing amounts of glucose remained in the fermentation broth when higher initial cassava starch concentrations were supplied. The highest production yield of 0.91 g g⁻¹ was reached with the initial cassava starch concentration of 70 g l⁻¹ with poor residual glucose remaining (8.40±0.43 g l⁻¹ succinate) compared to high residual glucose between 32-83 g l⁻¹ from initial cassava starch providing from 90-150 g l⁻¹. It was likely that high residual glucose concentration liberated from cassava starch might inhibit the succinate production and sugar utilization. These results were in accordance with the works of Chan and colleagues²¹ and Agarwal and colleagues²² in which the productions of succinate were performed in *E. coli* using high concentrations of sucrose and sugarcane molasses respectively. In addition, Wang and colleagues²³ proposed that the inhibition of high sugar concentration on the cell growth may probably result from a fall in water activity affecting the microbial metabolic rate. Zhu and colleagues²⁴ also pointed out that ethanol concentrations and yields by *Saccharomyces*

cerevisiae significantly decreased when cassava pulp concentrations were provided to the medium during the SSF process. Furthermore, Wang and colleagues²³ studied L-lactic acid production from cassava powder by *Lactobacillus rhamnosus* under the SSF process. They found that a high amount of crude cassava powder provided to the bioreactor partly inhibited the cell growth and caused lower L-lactic acid production. They also suggested that some toxic components including cyanides commonly found in cassava thus adversely affected the cell growth and microbial biochemical productions.

The maintenance of medium at high pH usually involves additions of higher amounts of base solution. However, **these additions increased costs**. In our experiment, no significant effect on yields and titers was observed when the pH was increased from 6.5 to 7.0. Therefore, the lower pH at 6.5 was preferred for the process, and was applied for further investigation. This phenomenon was also observed during succinate and other biochemical productions during the SSF process. Recently, Sawisit and colleagues²⁵ utilized cassava pulp with cellulase enzyme to obtain fermentable sugars during succinate production using the SSF process by the strain KJ122. They found that sugars were left over at higher concentrations at pH lower than 6.5. However, no significant difference of succinate titers and yields was found at pH between 6.5 and 7.0. In addition, Wang and colleagues²³ investigated an initial pH value in SSF of cassava powder for 2,3-Butanediol production by *Enterobacter cloacae* subsp. They also found that the bacterium started utilizing crude cassava powder and producing 2,3-butanediol at the highest level at pH about 6.0-6.5. Agarwal and colleagues²² also investigated a cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by wild type *E. coli*. The results revealed that pH is the important factor affecting both cell growth and growth-associated

production of succinate. They reported that the optimal pH for succinic acid production was also 6.5.

Finding an optimal enzyme loading is essential in the SSF process to achieve high titer of microbial production, yield, and productivity, and to reduce the cost of production by lowering the amount of enzyme dosage. In Figure 3, the use of enzyme loading between 500-1,300 U AMG g⁻¹ cassava starch did not influence titers and production yields of succinate by the strain KJ122. In addition, providing low amount of AMG at 300 U g⁻¹ cassava starch resulted in decreasing succinate titer and yield. Thus, a suitable AMG loading of 500 U g⁻¹ cassava starch was considered as an optimum for succinate production by *E. coli* KJ122 in the SSF from cassava starch. Chen and colleagues¹³ investigated the optimal pectinase loading for hydrolysis of acid-pretreated rapeseed meal in SSF for succinate production using *Actinobacillus succinogenes*. They found that the increased level of pectinase loading from 1% to 3% (v w⁻¹) had no influence on succinate concentration and its production yield. Furthermore, Wang and colleagues²³ also revealed that the utilization of AMG at the dosage of 400 U g⁻¹ cassava powder could completely release glucose from cassava powder within 4 h and resulted in the highest production of 2,3-butanediol by *En. cloacae*. In contrast, while an enzyme loading higher or lower than 400 U AMG g⁻¹ powder had a negative effect on 2,3-butanediol production by the strain in the SSF process. Sawisit and colleagues²⁵ also found that the highest efficacy of cassava pulp hydrolysis with cellulase complex loading at 3% (v w⁻¹) on dry pulp combined with 2 % (v w⁻¹ on dry pulp) AMG was achieved during succinate production by the strain KJ122 in SSF. No further improvement in sugars and succinate

productions from cassava pulp was observed when more concentrations of cellulase complex

were provided. In addition, it is not necessary to utilize an excessive level of enzyme in the SSF process resulting in a reduction of the cost of microbial biochemical production.

The balance of temperatures for fermentation and enzyme hydrolysis is also a key factor for many microbial biochemical productions using the SSF process.²⁴ Optimal temperatures for microbial growth and enzymatic activity used for substrate utilization should be compromised. In our study, the range of temperature between 37 and 41°C had no effect on the growth and succinate production of the strain KJ122. But the temperature at 43°C adversely affected the growth and succinate production by the strain. Additionally, at 37°C the strain KJ122 seemed to consume glucose at a slower rate than those at temperatures of 39°C and 41°C. Besides, no significant differences in succinate concentration and yield were observed when *E. coli* KJ122 was cultured at both temperatures of 39°C and 41°C. In the economy point of view, it is more suitable to operate the microbial cultivation at lower temperatures as the higher the temperature; the more the electrical power consumption.

Therefore, it was likely that succinate production from cassava starch at 39 °C would be satisfied by *E. coli* KJ122 during the SSF process. Our result was similar to other published works. Sawisit and colleagues²⁵ also performed succinate production from cassava pulp using the SSF process. They revealed that no significant differences in succinate titers and yields were observed when the fermentation was performed at temperatures ranging from 37 to 41°C. Zhu and colleagues²⁴ and Öhgren and colleagues²⁶ also demonstrated that neither a decrease nor an increase in the temperatures ranging from 30 to 40°C induced a significant decrease in ethanol production efficiency from cassava pulp by *Saccharomyces cerevisiae*. Moreover, Zheng and colleagues²⁷ conducted succinate production from corn stover under the SSF process by *Actinobacillus succinogenes* at temperatures in the range of 38 to 42°C.

Their result showed that with an increase in temperature over 38°C, both succinate concentrations and yields significantly decreased. In addition, Martinez and colleagues²⁸ observed no remarkable differences in yield, productivity, and final titer of succinate production by *E. coli* SBS550MG at temperatures ranging from 37 to 42°C.

During batch fermentation in a 2l fermentor, *E. coli* KJ122 could produce succinate at an impressive titer and yield of $70.1 \pm 0.1 \text{ g l}^{-1}$ and $1.01 \pm 0.01 \text{ g g}^{-1}$ glucose utilized respectively, with the overall productivity of $0.97 \pm 0.01 \text{ g l}^{-1} \text{ h}^{-1}$. The succinate yield and productivity obtained from cassava starch were in agreement with findings from glucose by the strain KJ122.⁶ Jantama and colleagues⁶ also revealed that the theoretical yield of succinate production by microbial fermentation from glucose was $1.71 \text{ mol mol}^{-1}$ glucose (1.12 g g^{-1} glucose). The strain KJ122 produced succinate at the yield of $1.4\text{-}1.5 \text{ mol mol}^{-1}$ glucose used. In this present study, the yield of succinate production from cassava starch by the strain KJ122 was achieved at $1.01 \pm 0.01 \text{ g g}^{-1}$ cassava starch utilized, which was equivalent to $1.39 \text{ mol mol}^{-1}$ glucose used (1g cassava starch is equivalent to 1.1 g glucose). Therefore, the theoretical succinate production yield of 81.29% was achieved. This indicated no significant difference in the succinate production yield from glucose and cassava starch produced by the strain KJ122 in batch fermentation. In addition, the specific productivity of $423 \text{ mg g}^{-1} \text{ CDW h}^{-1}$ was obtained by the strain KJ122 (Table 1). Chen and colleagues²⁹ revealed that the highest specific productivity of $171 \text{ mg g}^{-1} \text{ CDW h}^{-1}$ was achieved when glucose was used as carbon sources by *E. coli* NZN111. Sawisit and colleagues²⁵ also found that the specific productivity of succinate of $235 \text{ mg g}^{-1} \text{ CDW h}^{-1}$ was reached from batch fermentation of cassava pulp by the strain KJ122. The results suggested that *E. coli* KJ122 exhibited high efficiency in the specific productivity of succinate from glucose-based and

even cassava-derived substrates during batch fermentation compared to those previously reported works.

The fed-batch mode improves the viability of the microorganism due to increases in the efficiencies of mass and heat transfer, causing the hydrolysis and fermentation reaction to be faster and more efficient. Our results showed that the fed-batch SSF procedure also considerably increased yield, productivity, and succinate concentration by about 7.9%, 18.6%, and 18.4% respectively, compared to those of the batch SSF process. Chen and colleagues¹³ enhanced the succinic acid concentration from 15.5 g l⁻¹ to 23.4 g l⁻¹ at the yield of 11.5 g per 100 g dry matter and productivity from 0.22 to 0.33 g l⁻¹ h⁻¹ by utilizing the fed-batch SSF procedure from rapeseed meal by *A. succinogenes* strain. Additionally, Bretz and Kabasci³⁰ increased the succinate production yield by *An. succiniciproducens* from 0.60 to 0.88 g g⁻¹ glucose during fed-batch fermentation in comparison to the batch process performed with similar glucose concentrations. Sawisit and colleagues²⁵ also mentioned that higher succinate concentration and average productivity from cassava pulp were observed during fed-batch SSF compared to those found in batch experiments by the *E. coli* KJ122. Therefore, the results confirmed that succinate concentration, yield, and productivity could be efficiently improved by the fed-batch process compared to the batch SSF process.

In this present study, the yield of succinate production obtained from fed-batch SSF was equivalent to 1.03 g g⁻¹ cassava starch or 1.66 mol mol⁻¹ glucose used. Therefore, the theoretical succinate production yield of 92% was achieved. Chen and colleagues²⁹ produced succinate at high concentrations by high cell density cultures of *E. coli* NZN111 in rich medium containing cassava powder and starch. Succinate yields of 0.6-0.7 g g⁻¹ substrates obtained from their processes were significantly lower than those obtained in our study

(Table 1). In addition, lower specific productivities (259 and $339 \text{ mg g}^{-1} \text{ CDW h}^{-1}$ for cassava powder and starch respectively) were clearly observed during succinate production in the study of Chen and colleagues²⁹. In contrast, very high specific productivity of $456 \text{ mg g}^{-1} \text{ CDW h}^{-1}$ by the strain KJ122 from cassava starch was observed in our study.

Thailand is the world's third leading country in the production and export of cassava starch and about 2 million tons are produced annually in the country.⁸ Cassava is sold at a very low price (about $\$0.4/\text{kg}$ starch) and the use of this material is not competitive for human consumption. AMG and Amy enzymes cost $\$7.57/\text{kg}$ and $\$3.50/\text{kg}$ respectively per manufacturer's price. Also, the cost of AM1 medium is estimated at around $\$0.42/\text{L}$. Based on our study, the production cost of succinate was estimated at approximately $\$4.54\text{--}4.58/\text{kg}$ succinate. However, the cost of succinate produced by chemical synthesis is $\$5.9\text{--}8.8/\text{kg}$.¹ In addition; Sawisit and colleagues²⁵ recently proposed that the price of succinate produced by microbial fermentation from cassava pulp was about $\$5.21\text{--}5.45/\text{kg}$ succinate. It is likely that the utilization of cassava starch may make the microbial succinate production by *E. coli* KJ122 more economically competitive. Consequently, this may turn low-value agricultural products into high-value-added chemicals.

CONCLUSION

Succinate was produced with impressive titers, yields, and specific productivities by *E. coli* KJ122 under simple anaerobic conditions in a low-cost medium (AM1) containing only cassava starch using batch and fed-batch SSF and it compared favorably with other previously published works (Table 1). Our fermentation conditions differed from previous

reports in the use of inorganic salt mediums rather than complex media, the lack of carbon dioxide gas and auxotrophic requirements, and the use of simple batch operations under anaerobic conditions. Growth and succinate production occurred concurrently in a single step. Fermentation pH was controlled using a mixture of potassium carbonate and potassium hydroxide. These conditions would be expected to lower the costs of succinate production and purification, and waste disposal by the strain from cassava starch. Therefore, *E. coli* KJ122 would be one of the microbial strains for the cost-effective production of succinate from cassava starch as a sole carbon source.

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Table 1 Comparison of succinic acid production from cassava derived substrates with other previously published works

Substrate	Microorganism	Fermentation strategy	Time (h)	Cell concentration (g CDW l ⁻¹)	Succinate Production*				References
					Concentration (g l ⁻¹)	Yield (g g ⁻¹)	Productivity (g l ⁻¹ h ⁻¹)	Specific productivity (mg gCDW ⁻¹ h ⁻¹)	
Cassava powder	<i>E. coli</i> NZN111	Fed-batch SSF	73	10.1	106	0.66	2.54	259	[26]
Cassava starch	<i>E. coli</i> NZN111	Fed-batch SSF	73	17.0	127	0.71	1.77	339	[26]
Cassava pulp	<i>E. coli</i> KJ122	Batch SSF	96	3.58	80.9±0.5	0.70±0.03	0.84±0.01	235	[22]
		Fed-batch SSF		Not reported	98.6±0.1	0.72±0.04	1.03±0.01	Not reported	
Cassava starch	<i>E. coli</i> KJ122	Batch SSF	72	2.30±0.01	70.1±0.1	1.00±0.01	0.97±0.01	423	This study
		Fed-batch SSF		2.51±0.01	82.5±0.7	1.03±0.01	1.15±0.01	456	

* Yield was calculated as product concentration divided by utilizing substrate concentration during fermentation. Productivity was calculated as product concentration divided by overall fermentation time. Specific productivity was calculated as succinate productivity divided by cell dry weight concentration.

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Author/s:

Khor, K; Sawisit, A; Chan, S; Kanchanatawee, S; Jantama, SS; Jantama, K

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