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Contributed Paper

# Optimization of Succinic Acid Production by *Actinobacillus* sp. NP9-aA7 using Plackett-Burman Design Coupled with Box-Behnken Design

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## ABSTRACT

The potential succinic acid bacterium, isolate NP9-aA7, was isolated from bovine rumen fluid. The phenotypic characterization and phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate NP9-aA7 was identified to *Actinobacillus succinogenes*. The statistical methods combining Plackett-Burman Design (PBD) and Box-Behnken Design (BBD) were developed to optimize the process. The key factors consist of glucose 74 g/L, yeast extract 30 g/L, and $MgCO_3$ 60 g/L which gave a maximum succinic acid to 60.09 g/L with a yield of 0.8161 g/g glucose after 36 h. Batch fermentations in a 2-L fermenter leading to the highest succinic acid production which validated the model with the succinic acid of 58.08 g/L after 27 h. Moreover, the $CO_2$ of 50.66 kPa and alkaline neutralizers (45 g/L of $MgCO_3$ and 15 g/L of $Mg(OH)_2$) enhanced succinic acid to 72.93 g/L with a yield of 1.393 g/g glucose after 24 h. When using SSH 40 g/L as a carbon source, the succinic acid of 27.70 g/L was obtained after 48 h. *A. succinogenes* NP9-aA7 has a potential for succinic acid production and combined statistical methods were powerful for optimizing the process.

**Keywords:** *Actinobacillus* sp. NP9-aA7, Box-Behnken Design (BBD), carbon dioxide ($CO_2$), Plackett-Burman design (PBD), succinic acid

## 1. INTRODUCTION

Succinic acid has multitudinous applications. While the succinic acid production from petrochemical process has the limitation is high conversion cost and negatively affects the environment. Currently, succinic acid production from the fermentation process by microorganism is on the rise. It is an intermediate product of the tricarboxylic acid (TCA) pathway while it is an end-product of anaerobic metabolism. The production of succinic acid by Gram-positive bacteria such as *Corynebacterium*

*glutamicum* [1], *Enterococcus* species, *Lactococcus* species, and *Lactobacillus* species [2, 3] and Gram-negative bacteria such as *Actinobacillus succinogenes* [4], *Anaerobiospirillum succiniciproducens* [5], *Mannheimia succiniciproducens* [6] *Bacteroides fragile*[7], *Escherichia coli* [8], *Propionibacterium* sp., and *Ruminococcus flavefaciens* [9] have been reported. Most bacteria have been isolated from the various anaerobic environments such as domestic sludge, cow dung, dog's oral cavity, rumen, and gastrointestinal tract [10].

Most potential succinic acid producers are in the family of Pasteurellaceae including *A. succinogenes* ATCC 55618 [11], *A. succiniciproducts* ATCC 53488 [5], and *M. succiniciproducts* MBEL55E [6]. They were screened from bovine rumen and produced succinic acid as a major fermentation product of 52.70, 34.40 and 13.50 g/L, respectively. Lee et al. [12] reported that the promising succinic acid producers were isolated from ruminant rumen because the rumen gave a specific environment offer (such as carbon dioxide, methane and traces of hydrogen production).

However, there are many factors involved in the enhancement of succinic acid production such as the sources and amount of carbon, nitrogen, and neutralizing agent [13, 14]. Theoretically, succinic acid can be produced from glucose by *A. succinogenes* with following stoichiometry [12]

$$C_6H_{12}O_6 + 2CO_2 + 4H \rightarrow 2C_4H_6O_4 + 2H_2O$$

The supplies of $CO_2$ and electron donors are necessary to achieve high succinic acid production. Carbon dioxide could significantly affect the cell growth of anaerobic bacteria and include into the backbone of three carbon compound to generate four carbon of oxaloacetate through PEP carboxylase to promote the production [15, 16]. Therefore, the level of $CO_2$ in culture medium could effect on metabolic fluxes of fermentation products. Coenzymes and cofactors (biotin and magnesium ions) were also significantly affected on key enzymes in metabolic pathway [14] .

Therefore, a lot of experiments run concurrently with the interactions among various investigated factors. For fermentation medium optimization, Plackett-Burman Design (PBD) is an appropriate method for rapid screening of many key medium components and to identify the most significant independent factors [17]. The concentration of the key medium is optimized by Box-Behnken Design (BBD) using Response Surface Methodology (RSM) for estimation of the relationships between the response and the key factors. It has the advantages of reduction of the number of experiments and improving variables interaction analysis compared to the one-factor-at-a-time method [11].

This study aims to characterize and identify the succinic acid producing bacteria isolated from Thailand, and the production process is optimized using statistical methods.

## 2. MATERIALS AND METHODS

### 2.1 Isolation and Identification of Succinic Acid Bacteria

Bacterial strains were isolated from 3 samples of bovine rumen fluid from the Faculty of Agricultural, Kasetsart University, Kamphaeng Saen Campus, Nakornprathom province, Thailand, based on the method of Phuengjayaem et al. [3]. The potential succinic acid producing strain (Isolate NP9-aA7; LC192793) was characterized according to cell morphology (cell form, cell arrangement, and Gram reaction) and colony appearance (color, shape, margin, optical property, and elevation). The identification methods have been described by Sitdhipol et al. [18].

The physiological, including various pH values from 3.5 to 9.0, temperatures in the range of 20-50 °C, and concentration of NaCl (6.0% w/v NaCl) were tested using MRS broth (Difco, France). The biochemical characteristics were examined by a catalase test, gas production, arginine hydrolysis, nitrate reduction, starch hydrolysis, slime formation, and acid formation from various carbohydrates as described by Tanasupawat et al. [19]. The 16S rRNA gene of isolates was PCR amplified, the procedure of 16S rRNA gene sequence and phylogenetic analysis have been described by Phuengjayaem et al. [3]. The sequence of the 16S rRNA gene of isolate NP9-aA7 has been deposited in the DNA Data Bank of Japan (DDBJ) database.

**2.2 Succinic Acid Production**

The inoculum consists of 50 ml of 3.0% tryptic soy broth (TSB) medium (Difco, France). The inoculated cultures were cultivated at 37 °C, 200 rpm under anaerobic conditions for 24 h. The fermentation was conducted in a 250 ml flask with 50 ml of the production medium containing (g/L) yeast extract, 30; urea, 2.0, $MgCl_2.6H_2O$, 2.0; $CaCl_2$, 1.5; $MnCl_2$, 0.070; $Na_2HPO_4$, 4.4; $NaH_2PO_4$, 3.3; $MgCO_3$, 30 and the pH was adjusted to 7.0 [20]. Biotin 0.30 µg/L and thiamin 0.20 µg/L were added after being sterilized by membrane filtration (0.22 µm, Millipore Express, Ireland). Glucose was separately sterilized at 115 °C for 20 minutes and added to the medium to maintain the initial concentration of 60 g/L. It was incubated at 37 °C with agitation at 200 rpm for 36 h with 10% v/v seed inoculum to give a starting $OD_{660}$ about 0.080. All chemicals were purchased from Merck (Merck, Germany) unless otherwise described.

**2.3 Plackett-Burman Design (PBD)**

The experiment using PBD with 12 trials and 8 variables including glucose (A), yeast extract (B), $CaCl_2$ (C), $MgCl_2$ (D), $MnCl_2$ (E), $Na_2HPO_4$ (F), $NaH_2PO_4$ (G), $MgCO_3$ (H) and three dummy variables (J, K, and L). They were implemented in two levels such as minimum coded as "-1" and maximum coded as "+1" with triplicates at the center point [21].

**2.4 Effects of Various Concentrations of Biotin**

Biotin was added to the production medium in the range of 0-200 µg/L.

**2.5 Effects of Different Nitrogen Sources**

To optimize the nitrogen sources such as (g/L), $(NH_4)_2SO_4$, 15; $NH_4Cl$, 10; $KNO_3$, 19; urea, 6.8; peptone, 24; beef extract, 30 and corn steep liquor (CSL) 4.8% v/v were compared with 30 g/L of yeast extract (in same percent equivalent).

**2.6 Effects of Different Alkaline Neutralizers**

The pH of the medium was regulated to pH 7.0 with the addition of varying alkaline neutralizers at 10 g/L such as $CaCO_3$, $Ca(OH)_2$, $Na_2CO_3$, $NaHCO_3$, NaOH, $MgCO_3$, and $Mg(OH)_2$.

**2.7 Response Surface Methodology (RSM)**

A Box-Behnken design (BBD) was performed to assess the effect of the key medium components on the response surface in the region of examination. The experimental design was composed of three variables (glucose (A), yeast extract (B), and $MgCO_3$ (C)) with three levels coded as 1, 0, and +1. The experiments were conducted using the

conventional 'one-factor-at-a-time' method to select the eligible factors for maximum succinic acid production.

The response surface analysis was based on multiple linear regressions that take into account the principal results, quadratic and interaction effects by the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_2 X_i X_j + \sum_{i=1}^{3} \beta_3 X_i^2 \quad (1)$$

Where Y is the predictive response. $X_1$, $X_2$, and $X_3$ are the parameter values for the independent variables. The constants $\beta_0$, $\beta_1$, $\beta_2$, and $\beta_3$, are coefficient estimates for succinic acid production. The constant $\beta_0$ is the intercept term, $\beta_1$ is the liner effect, $\beta_2$ is the interaction, and $\beta_3$ is the quadratic effect. The process performance was estimated by analyzing the succinic acid concentration produced after 36 h of cultivation time. For optimization, the response can involve independent variables based on the quadratic models. The result from the statistical analysis, including analysis of variance (the interactive results between variables and response) and the quality of fit of the quadratic equation were represented by the coefficient of determination $R^2$ and the *F*-test.

### 2.8 Determination of the Optimum Ratio of Alkaline Neutralizers

The effects of the different alkaline neutralizer ratios at 3:1, 2:1, 1:1, 1:2 and 1:3 of $MgCO_3$: $Mg(OH)_2$ used to control pH were investigated.

### 2.9 Utilization of SSH as a Carbon Source for Succinic Acid Fermentation

The fermentation process was performed using various concentrations in the range 0-80 g/L SSH. The preparation of SSH was described by Poonsrisawat et al. [22].

### 2.10 Succinic Acid Fermentation by *A. succinogenes* NP9-aA7 in a 2-L Fermenter

The optimal of medium composition as above were verified in a 2-L fermenter (B.E. Marubishi Co., Inc., Thailand) containing 1.2 L of fermentation medium, as well as the initial carbon sources was 74 g/L of glucose or 40 g/L of SSH. The pH was controlled at 7.0 with 5.0 N NaOH and 5.0 N $H_3PO_4$ and temperature was maintained at 37 °C, 200 rpm for 48 h (take the sample every 3 h) with 10% (v/v) inoculated of preculture broth and external $CO_2$ gas sparging rate was 1.0 vvm. $CO_2$ and $N_2$ were from linde industrial gases, Thailand (Linde, Thailand). Foam was controlled by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO).

### 2.11 Effects of $CO_2$ Partial Pressure with $MgCO_3$

The $CO_2$ partial pressure on succinic acid production was studied by various pressures at 25.33, 50.66, 75.99 and 101.3 kPa (100% $CO_2$ gas) in a 2-L fermenter.

### 2.12 Analytical Methods

Cell concentration was measured as optical density at wavelength 660 nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan) after removing the insoluble $MgCO_3$ in the sample by adding 0.2 M HCl [23]. The reducing sugars in the SSH were measured by DNS (3, 5-dinitrosalicylic acid colorimetric) method applied from Miller [24], with D-glucose as the standard. Fermentation products (succinic, acetic and formic acid) were analyzed with HPLC. The condition for HPLC analysis was described by Phuengjayaem et al. [3].

### 2.13 Statistical Analysis

All experiments were tested in triplicate, and the related data were expressed as

average values. The statistical software package "Minitab 17" was used to analyze the results from the PBD experimental data. Statistical Package for the Social Sciences (SPSS) for Windows program version 15 was used to analyze the data from one-factor-at-a-time (the effect of various biotin concentrations, different nitrogen sources, different alkaline neutralizers, the ratio of alkaline neutralizer, various SSH concentrations used as a carbon source, and $CO_2$ partial pressure). Firstly, SPSS analysis (one-way ANOVA method) was used for comparison of each factors then Duncan's and Tukey's multiple range test method were pairwise comparison tested. The statistical software package Design-Expert 6.0 (trial version) was used to analyze the results from BBD and carried out a regression analysis for the equations and an estimation of the statistical significance of the different quadratic equation.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation and Identification of Succinic Acid Bacteria

From primary screening, 49 isolates were re-streak on enrichment medium plate then transferred to the screening medium plate. Twenty five isolates exhibited a clear zone on the screening medium plate. Secondary screening, qualitative by TLC method, 19 isolates were able to produce succinic acid. Subsequently, quantitative by HPLC method, 14 isolates produced succinic acid more than 60% yield. The potential 6 isolates were representative for phenotypic characteristic. The criteria for selection the promising isolate were facultative anaerobe with high ability to produce succinic acid. *A. succinogenes* NP9-aA7 was selected for optimization succinic acid production. In addition the result from phenotypic characteristic, isolate NP9-aA7 could grow at pH 3.5 which benefit of acid tolerant property suitable to apply in acid fermentation process.

The isolate NP9-aA7 was a non-spore forming and Gram-negative rods. Colonies on the screening agar plate after 24 h of incubation were gray, punctiform, convex, translucent and the diameter of 1.0-1.5 mm. Moreover, it was capable of fermenting varieties of carbon sources such as D-amygdalin, L-arabinose, cellobiose, gluconate, glucose, lactose, D-mannitol, ribose, salicin, sorbitol, and D-xylose under anaerobic conditions but negative result of starch hydrolysis and slime test and other results were shown in Table 1. The 16S rRNA sequence (1,440 bases) was determined for isolate NP9-aA7 similar to *Actinobacillus succinogenes* 130z$^T$of the family Pasteurellaceae at 99.93% similarity. Therefore, this isolate was identified as *Actinobacillus succinogenes*. Subsequently, a phylogenetic tree was constructed as seen in Figure 1. The sequence of the 16S rRNA gene of isolate NP9-aA7 has been deposited in the DDBJ database under the accession number: LC192793.

### 3.2 Plackett-Burman Design (PBD)

As shown in Table 2, the results from PBD were assessed.). The maximum concentration of 27.38 g/L succinic acid was obtained from Run No. 10 (60 g/L glucose, 15 g/L yeast extract, 1.0 g/L $CaCl_2$, 3.0 g/L $MgCl_2$, 0.10 g/L $MnCl_2$, 1.5 g/L $Na_2HPO_4$, 1.0 g/L $NaH_2PO_4$, and 45 g/L $MgCO_3$) while productivity and cell growth were 0.7610 g/L·h and $OD_{660}$ 5.630, respectively. The bar graph shows the sorting of the higher effect from top to bottom (Figure 2). The chart contains a vertical line at critical *t*-value (at $\alpha$ 0.05000) when the bar greater than the vertical line was accepted while the bar smaller than the vertical line was considered not significant and that variable was not accepted. The result indicated that glucose, yeast extract, and $MgCO_3$ had a

confidence level higher than 95%. These factors were concluded as the significant effect of succinic acid production. A first-order regression equation is shown in Equation 2 from the PDB analysis:

$$Y = 13.20 + 3.150\ A + 2.855\ B - 0.3910\ C + 0.2190\ D - 0.3430\ E + 0.5300\ F - 1.920\ G + 3.666\ H \quad (2)$$

Where Y is the succinic acid concentration, A, B, C, D, E, F, G, and H are the values of glucose, yeast extract, $CaCl_2$, $MgCl_2$, $MnCl_2$, $Na_2HPO_4$, $NaH_2PO_4$, and $MgCO_3$, respectively, while J, K, and L are dummy variables.

Succinic acid production was affected by the coefficients of glucose (3.150), yeast extract (2.855), and $MgCO_3$ (3.666). The positive coefficient demonstrated that the high concentration of glucose, yeast extract, and $MgCO_3$ enhanced the amount of succinic acid. The optimal concentrations of these key factors are further studied using RSM.

**Table 1.** Comparison of phenotypic characteristics of isolates NP9-aA7 and $130Z^T$.

| Characteristics | NP9-aA7 | $130Z^T$* | Characteristics | NP9-aA7 | $130Z^T$* |
|---|---|---|---|---|---|
| Cell form | Rods | Rods | Acid from: | | |
| Arginine hydrolysis | - | - | Gluconate | + | + |
| Gas from glucose | - | - | Glucose | + | + |
| Catalase | + | + | Lactose | + | + |
| Nitrate reduction | + | + | Maltose | - | + |
| Growth in 6% NaCl | + | - | D-Mannitol | + | + |
| Growth at pH 3.5 | + | - | D-Mannose | - | + |
| pH 5.0 | + | 6.0-7.4 | Melibiose | - | - |
| pH 9.0 | - | - | ∞-Methyl-D- | - | - |
| 20 °C | - | - | glucoside | | |
| 40 °C | + | 37 | Raffinose | - | + |
| 50 °C | - | - | Rhamnose | - | - |
| Acid from: | | | Ribose | + | + |
| D-Amygdalin | + | + | Salicin | + | + |
| L-Arabinose | + | + | Sorbitol | + | + |
| Cellobiose | + | + | Sucrose | - | + |
| D-Fructose | - | + | Trehalose | - | - |
| D-Galactose | - | + | D-Xylose | + | + |

+, Positive reaction; -, negative reaction. *Data from Guettler et. al., 1999.

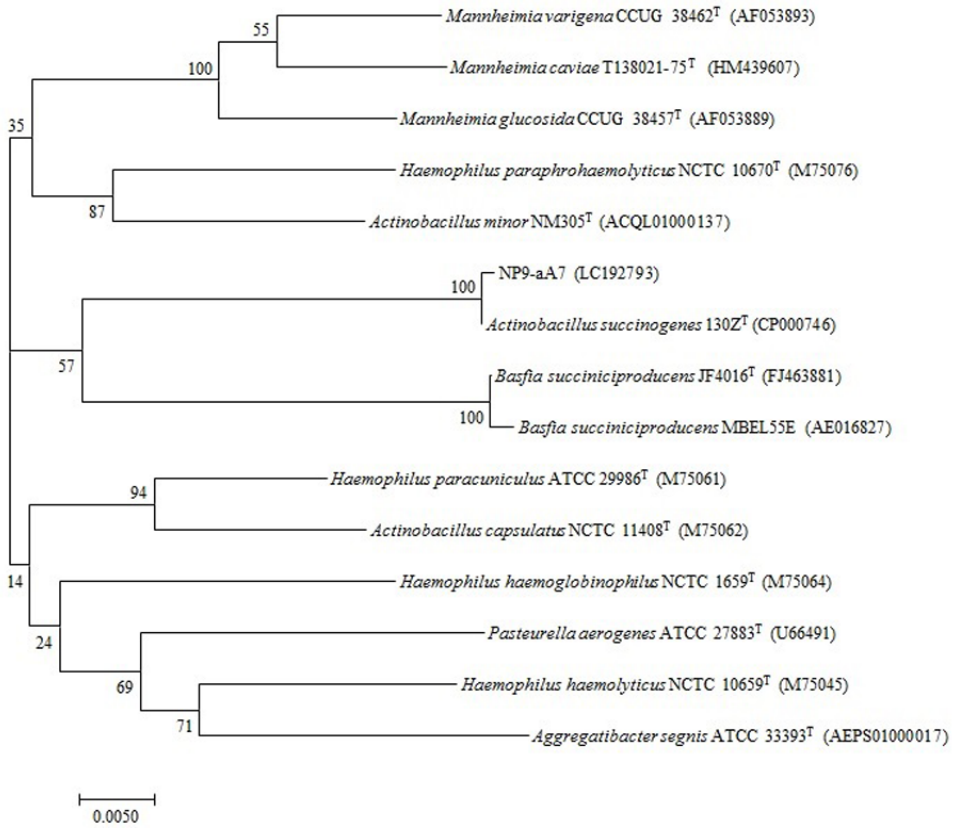


**Figure 1.** Neighbor-joining phylogenetic tree of isolates NP9-aA7 based on 16S rRNA gene sequences. Bootstrap values were expressed as percentages of 1000 replications.

**Table 2.** Experimental design using PBD and the results for the optimization of succinic acid production by *A. succinogenes* NP9-aA7.

| Run No. | A | B | C | D | E | F | G | H[a] | Cell growth ($OD_{660}$) | Succinic acid (g/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Actual | Predicted |
| 1 | 60(1) | 15(1) | 3(1) | 1(-1) | 0.3(1) | 4.5(1) | 1(-1) | 45(1) | 9.660 | 24.46 | 24.38 |
| 2 | 20(-1) | 15(1) | 1(-1) | 1(-1) | 0.1(-1) | 4.5(1) | 3(1) | 45(1) | 12.98 | 14.47 | 15.71 |
| 3 | 20(-1) | 5(-1) | 1(-1) | 1(-1) | 0.1(-1) | 1.5(-1) | 1(-1) | 15(-1) | 6.555 | 5.527 | 5.442 |
| 4 | 60(1) | 5(-1) | 3(1) | 3(1) | 0.1(-1) | 4.5(1) | 1(-1) | 15(-1) | 4.880 | 11.23 | 12.46 |
| 5 | 60(1) | 15(1) | 1(-1) | 3(1) | 0.3(1) | 1.5(-1) | 3(1) | 15(-1) | 5.285 | 11.21 | 13.37 |
| 6 | 20(-1) | 5(-1) | 3(1) | 3(1) | 0.3(1) | 1.5(-1) | 3(1) | 45(1) | 6.500 | 8.913 | 7.905 |
| 7 | 20(-1) | 5(-1) | 1(-1) | 3(1) | 0.3(1) | 4.5(1) | 1(-1) | 45(1) | 7.590 | 12.58 | 13.59 |
| 8 | 60(1) | 5(-1) | 3(1) | 1(-1) | 0.1(-1) | 1.5(-1) | 3(1) | 45(1) | 3.450 | 13.44 | 14.45 |
| 9 | 20(-1) | 15(1) | 3(1) | 1(-1) | 0.3(1) | 1.5(-1) | 1(-1) | 15(-1) | 5.440 | 9.599 | 9.684 |
| 10 | 60(1) | 15(1) | 1(-1) | 3(1) | 0.1(-1) | 1.5(-1) | 1(-1) | 45(1) | 5.630 | 27.38 | 25.22 |
| 11 | 20(-1) | 15(1) | 3(1) | 3(1) | 0.1(-1) | 4.5(1) | 3(1) | 15(-1) | 4.245 | 9.263 | 8.030 |
| 12 | 60(1) | 5(-1) | 1(-1) | 1(-1) | 0.3(1) | 4.5(1) | 3(1) | 15(-1) | 3.945 | 10.43 | 8.277 |

[a] 3 Dummy variables data are not shown.

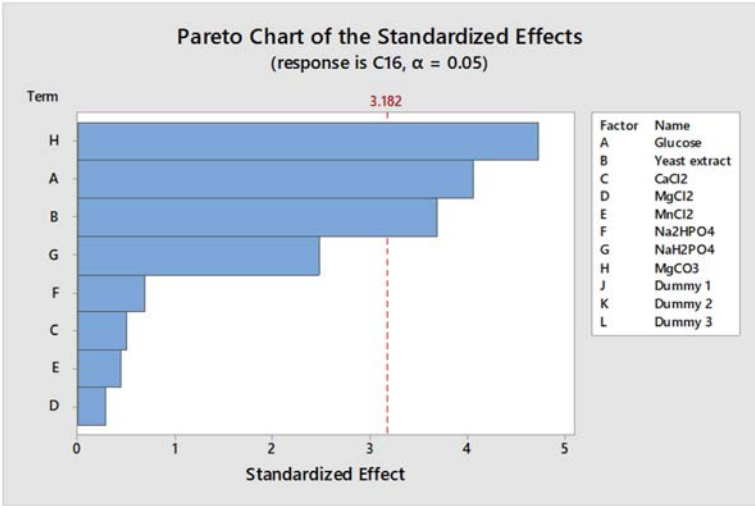


**Figure 2.** Pareto chart of standardized effects on succinic acid production. The chart includes a vertical line (i.e., standardized effect = 4.159) at the critical *t*-value for α of 0.05. The bars are displayed in order of the size of the impacts, and the standardized effect of each term is shown on the top of its corresponding bar.

The phenomenon that occurs in this experiment was explained that glucose was used as a carbon source, it was essential for cell synthesis and succinic acid production. Yeast extract affected cell growth directly. It contains many trace elements such as folic acid, pantothenic acid, biotin, and vitamin B1, B2, B6, and B12. This may explain why many kinds of vitamins could be omitted in the medium [11]. While $MgCO_3$ was used as an alkaline neutralizer for the control of the pH. Furthermore, the addition of $CO_2$ to the fermentation medium fundamentally affected the metabolic flux of carbon and the action of phosphoenolpyruvate carboxykinase (PEPCK) which were the prominent steps for succinic acid production [25]. In the *A. succinogenes*'s fermentation, $CO_2$ donor and $MgCO_3$ interact with organic acids in the medium resulting in the dissolved concentrations of $HCO_3^-$, $CO_3^{2-}$, and $CO_2$ were increased. However, insoluble $MgCO_3$ caused an opaque culture medium, which caused the cells to be evenly distributed in the medium broth. This helps to avoid cell flocculation. All these features make $MgCO_3$, one of the key factors which significantly improve the amount of succinic acid. Consequently, the variables of glucose, yeast extract and $MgCO_3$ have a significant effect on succinic acid production. Further study is required to determine the optimal concentrations of the three key factors.

### 3.3 Effects of Various Concentrations of Biotin

Succinic acid fermentation by *A. succinogenes* NP9-aA7 was increased from 14.53 g/L (without added biotin) to 44.19 g/L (50 µg/L of biotin) which moreover improved to 48.36 g/L with the addition of 0.20 mg/L biotin (Table 3). The statistical analysis using Duncan's method for multiple comparisons showed that at levels of 50 µg/L to 0.20 mg/L biotin produced succinic acid in the range of 44.19-48.36 g/L and was not significant (*p*-value of 0.06136). Moreover, biotin concentration could be used to reduce the demand for yeast extract to promote the

amount of succinic acid. It can be concluded that 50 µg/L biotin was optimal for succinic acid fermentation by *A. succinogenes* NP9-aA7. Similar to the research of Xi et al. [26], adding 50 µg/L to 0.10 mg/L of biotin could be improve succinic acid to 45.20 g/L in 3 L fermenter.

**Table 3.** The Summary of the results of each experiment for optimal succinic acid production by *A. succinogenes* NP9-aA7.

| Factors | 50 µg/L Biotin | Yeast extract | $MgCO_3$ | 3:1 of $MgCO_3$:$Mg(OH)_2$ | 40 g/L SSH |
|---|---|---|---|---|---|
| Residual glucose (g/L) | 0.03533±[c] 0.008266 | 0.1030± 0.002000 | 0.1119± 0.02466 | 0.196± 0.1798 | 6.132± 0.4352 |
| Glucose utilization (%)[a] | 99.94± 0.01378 | 99.83± 0.003333 | 99.81± 0.04110 | 99.67± 0.2996 | 83.22± 1.191 |
| $OD_{660}$ | 14.57± 0.9482 | 14.90± 1.746 | 5.760± 0.3011 | 15.14± 2.321 | 4.615± 0.1022 |
| Succinic (g/L) | 44.19± 5.073 | 49.76± 6.763 | 35.76± 1.776 | 48.64± 0.07300 | 19.14± 0.6847 |
| Formic (g/L) | 5.276± 0.3130 | 6.463± 0.1505 | 3.834± 0.3630 | 17.61± 10.64 | 2.797± 3.280 |
| Acetic (g/L) | 3.098± 0.2168 | 4.501± 0.3540 | 0.2967± 0.05850 | 4.481± 0.1010 | 0.6100± 0.05565 |
| Succinic acid yield (g/g glucose) | 0.7169± 0.06910 | 0.8307± 0.1129 | 0.5971± 0.02953 | 0.8132± 0.003151 | 0.6323± 0.02715 |
| Succinic acid productivity (g/L·h)[b] | 1.227± 0.1409 | 1.382± 0.2657 | 0.9932± 0.06978 | 1.351± 0.002868 | 0.5416± 0.01136 |

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate experiments.

The metabolic pathway of *A. succinogenes* converts glucose into phosphoenolpyruvate (PEP) then divides it into the two branches including: the acetate-producing C3 pathway and the succinate-producing C4 pathway. PEP is converted into fermentation products through the C3 pathway and the C4 pathway with malic enzyme and oxaloacetate (OAA) decarboxylase forming reversible shunts between these pathways. The higher biotin

concentration accommodates the PEP flow to the C4 pathway, while the flux of the C3 pathway is reduced. Biotin plays a key role in electron transfer in the anaerobic respiratory system of *A. succinogenes* [26].

### 3.4 Effects of Different Nitrogen Sources

The low cell growth was encountered with all the tested inorganic nitrogen sources. The case of organic nitrogen source, beef extract, and yeast extract was found to be the best nitrogen source for succinic acid fermentation by *A. succinogenes* NP9-aA7. The highest succinic acid of 51.03 g/L and cell growth of 13.43 ($OD_{660}$) were obtained when using 30 g/L beef extract followed by the succinic acid of 49.76 g/L and cell growth of 14.90 ($OD_{660}$) when using yeast extract (Table 3). From multiple comparisons of statistical analysis, the best groups, including beef extract and yeast extract, were found. Both sources of nitrogen should not be significantly different with a *p*-value of 0.9989 by the Duncan's method. However, the cost of the beef extract was higher than the yeast extract. From these results, it can be concluded that the use of yeast extract of 30 g/L was the suitable nitrogen source for the succinic acid fermentation by *A. succinogenes* NP9-aA7

Liu et al. [27] studied the effect of different nitrogen sources on cell growth and succinic acid fermentation by *A. succinogenes* CGMCC1593. They were reported that inorganic nitrogen sources were not efficient to promote cell growth of strain CGMCC1593 because they lack the amino acids and vitamins. Among the evaluated organic nitrogen sources, yeast extract gave the best results for both cell growth and succinic acid production. *A. succinogenes* CGMCC1593 gave a succinic acid of 48.00 g/L from yeast extract of 15 g/L which closely to our work.

### 3.5 Effects of Different Alkaline Neutralizers

The statistical analysis shows that the best group, including $MgCO_3$ and $Mg(OH)_2$, were not significantly different with a *p*-value of 0.05182 by Duncan's method. The maximum succinic acid of 35.76 g/L was obtained when using 10 g/L $MgCO_3$ as an alkaline neutralizing agent (Table 3).

Similarly, Liang et al. [20] reported on the effects of different alkaline neutralizers on cell growth and succinic acid fermentation by *E. coli* NZN111. The consumption of glucose in fermentation with $Na_2CO_3$, $NaHCO_3$, $Mg(OH)_2$, and $MgCO_3$ was much higher compared to $Ca(OH)_2$, $CaCO_3$, NaOH, and $NH_3H_2O$. The use of $MgCO_3$ caused the proper cell growth, glucose utilization, and succinic acid production. In addition, Li et al. [14] also reported that $MgCO_3$ was the best alkaline neutralizing agent for succinic acid production by *A. succinogene* NJ113 which gave a maximum succinic acid of 57.67 g/L. It was higher than this research because they used higher glucose concentration as a carbon source of 81 g/L.

$MgCO_3$ not only controls pH, but also play an essential role in the association of $CO_2$ with phosphoenolpyruvate carboxylase (PEPC) which stimulated the carboxylation of phosphoenolpyruvate to the origin C4 metabolite oxaloacetate [28]. Moreover, $Mg^{2+}$ was reported as the cofactor for another key enzyme in succinic acid pathway such as phosphoenolpyruvate carboxykinase (PEPCK) [25], pyruvate carboxylase (PC) [29] were an NADH dependent enzyme which catalyzes the conversion of oxaloacetate to malate [8].

In this case, NaOH was used as an alkaline neutralizer resulting not satisfactory. A high $Na^+$ level could result in a hypertonic environment which negatively affects the metabolism and cell growth [27]. Moreover,

$OH^-$ was a strong base. $Na^+$ also played an essential role in maintaining the transmembrane pH gradient, cell osmotic pressure, and regulation of intracellular pH. $Ca^{2+}$ can adapt to the natural fluidity and permeability of cell membranes which may affect the cell growth and cell metabolism resulting in low level of succinic acid when $Ca(OH)_2$ or $CaCO_3$ were used as alkaline neutralizers [30].

From this experiment, $Mg(OH)_2$ with strong alkalinity and high solubility and $MgCO_3$ for providing the $CO_3^{2-}$ were selected as an affordable mixed alkaline neutralizer to control pH for succinic acid fermentation by *A. succinogenes* NP9-aA7.

### 3.6 Response Surface Methodology (RSM)

The BBD with the RSM was used to determine the optimal medium concentration and the interactive effects of succinic acid fermentation by *A. succinogenes* NP9-aA7. Glucose, yeast extract, and $MgCO_3$ were selected as the factors for BBD. The concentrations of succinic acid for each run together with the predicted responses are shown in Table 4. The maximum succinic acid of 57.63 g/L was obtained when the concentrations of glucose, yeast extract, and $MgCO_3$ were 60, 30, and 60 g/L, respectively, with cell growth of ($OD_{660}$) 21.20 (Run 3). Based on the software analysis, the optimized concentrations of glucose, yeast extract, and $MgCO_3$ were 74 (0.74), 30 (1.0), and 60 (1.0) g/L and the predicted concentration of succinic acid was 57.63 g/L. This was a 15.83% improvement over that attained with the one-at-a-time method (49.86 g/L succinic acid was obtained from 30 g/L yeast extract as a nitrogen source with 30 g/L $MgCO_3$ as an alkaline neutralizer). The application of RSM plots the following regression equation, which was an empirical interaction between succinic acid and the variables in coded units:

$$Y = 39.89 - 1.307A + 10.42B + 3.904C + 15.17AB - 3.353AC + 6.323BC - 7.487A^2 - 7.042B^2 + 2.709C^2 \quad (3)$$

Where Y is the succinic acid produced as a function of glucose (A), yeast extract (B), and $MgCO_3$ (C). The statistical significance of the above equation was verified with the *F*-test and an ANOVA for the response surface quadratic model shown in Table 5.

The Model *F*-value of 17.97 indicates that the model was significant. There was only a 0.05% chance that a "Model *F*-Value" with this large value could occur because of noise. Values of probability (*P*) "Prob >*F*" of 4.830 $10^{-4}$ indicate that the model terms were significant. In this case, B, C, A2, B2, AB, BC were significant model terms. Values of probability higher than 0.05000 indicate the model terms were not significant. The "Lack of Fit *F*-value" was 3.510 implying the Lack of Fit was not significant relative to the pure error. There was a 12.83% chance that a "Lack of Fit *F*-value" this substantial value could occur due to noise. A not significant lack of fit indicates that the model was a good fit [31]. The fair precision value which measured the signal to noise ratio was 18.73. A ratio higher than four is satisfactory, so this model could be used to design the space. The quadratic equation shows that the $R^2$ value was 0.9585 which indicates opportuneness of the model. This result suggests that approximately 95.85% of the variability in the dependent variable can be explained by this model. The values of *R*-square more than 0.7500 indicate aptness of the model [32]. Therefore, the model was considered suitable to predict succinic acid production.

**Table 4.** Experimental design using Box-Behnken Design (BBD) and the results for the optimization of succinic acid production by *A. succinogenes* NP9-aA7.

| Run No. | Glucose | YE | $MgCO_3$ | Cell growth | Succinic acid (g/L) | |
|---|---|---|---|---|---|---|
| | A | B | C | ($OD_{660}$) | Actual | Predicted |
| 1 | 80(1)[a] | 30(1) | 40(0) | 18.04 | 51.53 | 49.64 |
| 2 | 60(0) | 20(0) | 40(0) | 14.03 | 35.77 | 39.89 |
| 3 | 60(0) | 30(1) | 60(1) | 21.20 | 57.63 | 56.19 |
| 4 | 40(-1) | 20(0) | 20(-1) | 12.87 | 32.48 | 29.16 |
| 5 | 60(0) | 20(0) | 40(0) | 13.97 | 39.90 | 39.89 |
| 6 | 40(-1) | 30(1) | 40(0) | 1.690 | 17.83 | 21.91 |
| 7 | 80(1) | 20(0) | 60(1) | 11.47 | 31.03 | 34.36 |
| 8 | 60(0) | 10(-1) | 60(1) | 8.210 | 21.96 | 22.72 |
| 9 | 40(-1) | 10(-1) | 40(0) | 10.82 | 29.53 | 31.42 |
| 10 | 60(0) | 20(0) | 40(0) | 16.00 | 38.90 | 39.89 |
| 11 | 80(1) | 20(0) | 20(-1) | 12.14 | 30.60 | 33.25 |
| 12 | 60(0) | 10(-1) | 20(-1) | 9.410 | 26.13 | 27.56 |
| 13 | 60(0) | 20(0) | 40(0) | 16.71 | 42.72 | 39.89 |
| 14 | 60(0) | 30(1) | 20(-1) | 15.42 | 36.51 | 35.74 |
| 15 | 80(0) | 10(-1) | 40(0) | 10.55 | 2.556 | -1.530 |
| 16 | 40(-1) | 20(0) | 60(1) | 17.720 | 46.33 | 43.68 |
| 17 | 60(0) | 20(0) | 40(0) | 14.597 | 42.16 | 39.89 |

YE, yeast extract

[a] Real values of the independent variables and the corresponding in parentheses mean code levels.

**Table 5.** Analysis of variance (ANOVA) for the Response Surface Quadratic Model.

| Source | Sum of Squares | DF[a] | Mean Square | F-Value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 2613 | 9 | 290.4 | 17.97 | $4.830\ 10^{-4}$ | significant |
| A | 13.66 | 1 | 13.66 | 0.8454 | $3.884\times10^{-1}$ | |
| B | 867.7 | 1 | 867.7 | 53.70 | $1.588\times10^{-4}$ | |
| C | 121.9 | 1 | 121.9 | 7.547 | $2.863\times10^{-2}$ | |
| A2 | 236.0 | 1 | 236.0 | 14.61 | $6.526\times10^{-3}$ | |
| B2 | 208.8 | 1 | 208.8 | 12.92 | $8.802\times10^{-3}$ | |
| C2 | 30.90 | 1 | 30.90 | 1.912 | $2.092\times10^{-1}$ | |
| AB | 920.4 | 1 | 920.4 | 56.96 | $1.320\times10^{-4}$ | |
| AC | 44.98 | 1 | 44.98 | 2.784 | $1.392\times10^{-1}$ | |
| BC | 159.9 | 1 | 159.9 | 9.898 | $1.623\times10^{-2}$ | |
| Residual | 113.1 | 7 | 16.16 | | | |
| Lack of Fit | 81.96 | 3 | 27.32 | 3.510 | $1.283\times10^{-1}$ | not significant |
| Pure Error | 31.14 | 4 | 7.785 | | | |
| Total | 2726 | 16 | | | | |
| $R^2 = 0.9585$, adj $R^2 = 0.9052$, Pred $R^2 = 0.5012$ | | | | | | |

[a] DF: degrees of freedom; $R^2$: determination coefficient; adj $R^2$: adjusted $R^2$; pred $R^2$: predicted $R^2$. Standard Deviation: 4.020, Mean 34.33, Coefficient of variation: 11.71, PRESS: 1360, Adeq Precision: 18.73.

The response surface was plotted to investigate the interactions between the factors and to determine the optimal concentration of key medium for maximum succinic acid production (Figure 3).

As shown in Figure 3a, the effects of glucose and yeast extract on the succinic acid production were determined when the other factor was at its center point although the increased glucose concentration did not increase succinic acid concentration when yeast extract was at a low level. Similar to cases of low levels of glucose (40 g/L), succinic acid did not increase with increasing yeast extract concentration.

Figure 3b shows that the succinic acid production was improved significantly by increasing the amount of glucose from 40 to 80 g/L with yeast extract at a center point (20 g/L). Adding enough $MgCO_3$ led to a higher succinic acid concentration when glucose was in the range of 50-70 g/L. Succinic acid production was not promoted with the low glucose level, although $MgCO_3$ was increasing. Therefore, both glucose and $MgCO_3$ were at high levels. Thus, more succinic acid was obtained.

Figure 3c illustrates the interaction of yeast extract and $MgCO_3$ on the concentration of succinic acid when glucose concentration was at its center point (60 g/L). It is precisely shown that succinic acid concentration was improved by increasing the amount of $MgCO_3$ with the amount of yeast extract added.

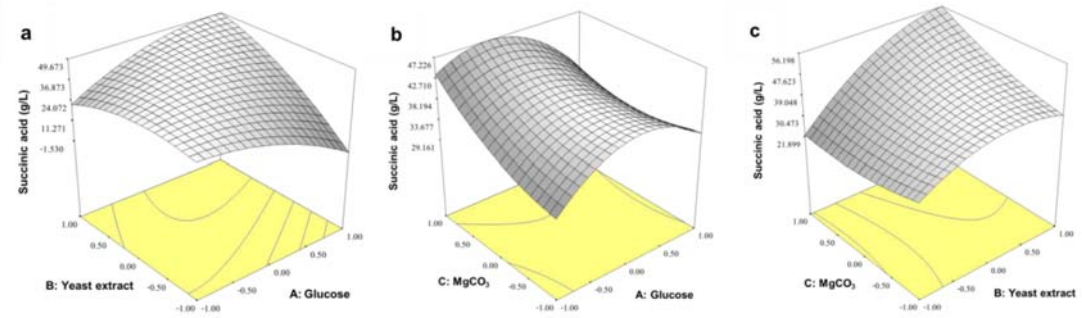


**Figure 3.** Response surface plots and contour plots.
(a) Combined effects of glucose and yeast extract with constant magnesium carbonate (40 g/L);
(b) Combined effects of glucose and magnesium carbonate with constant yeast extract (20 g/L);
(c) Combined effects of yeast extract and magnesium carbonate with constant glucose (60 g/L).

The statistical method using RSM gave a maximum succinic acid of 57.63 g/L. It was higher than the research of Zhu et al. [11]. They reported that the methods combining PBD, the steepest ascent method (SA) and the BBD were used to optimize succinic acid fermentation by *A. succinogenes* ATCC 55618. The results showed that glucose, yeast extract, and $MgCO_3$ were identified to be the key factors by PBD. Then, they preliminarily used the SA method, followed by the BBD method for the medium optimization. The maximal succinic acid of 52.70 g/L was obtained from 85, 14.5 and 64.7 g/L of glucose, yeast extract

and $MgCO_3$, respectively. Zhang et al. [33] used the model from RSM to predict the succinic acid fermentation by *A. succinogenes,* strain B-1. The glucose, yeast extract, and interactive effect of yeast extract and $MgCO_3$ were found to be the most significant effect on succinic acid production. The optimum values for glucose, yeast extract, and $MgCO_3$ concentrations were found to be 27.43, 9.560, and 23.32 g/L, respectively. This resulted in a predicted value of 19.08 g/L.

### 3.7 Model Validation

Based on the validation results of the combined levels of the three factors including glucose, yeast extract, and $MgCO_3$, were predicted, according to the quadratic equation from BBD. The applicability of the model and the accuracy of the prediction were verified based on triplicate experiments using the optimized conditions representing the maximum point of the concentration of succinic acid to confirm the results of the model (Table 6).

**Table 6.** Comparison between the actual and predicted succinic acid using a quadratic equation from BBD.

| No. | Glucose | YE[a] | $MgCO_3$ | Succinic acid | | | |
|---|---|---|---|---|---|---|---|
| | | | | Actual | Predicted | Residual (g/L) | Error (%) |
| 1 | 74.0 | 30.0 | 60.0 | 60.09 | 59.89 | 0.2014 | 0.3352 |
| 2 | 72.4 | 30.0 | 60.0 | 58.60 | 59.84 | -1.243 | 2.122 |
| 3 | 73.6 | 29.9 | 60.0 | 57.08 | 59.79 | -2.712 | 4.752 |
| 4 | 65.2 | 30.0 | 60.0 | 55.86 | 58.44 | -2.586 | 4.630 |
| 5 | 67.4 | 29.0 | 60.0 | 54.42 | 58.21 | -2.514 | 4.515 |

[a]YE, Yeast extract

Validation of the model was also conducted and the percentage of errors between the actual and predicted values for succinic acid production varied from 0.3352% to 4.515%. The predicted average concentration of succinic acid was 57.21 ± 2.227 g/L and the average concentration determined from the experiment was 59.23 ± 0.8345 g/L. These results show that there was not significant between actual and predicted values (3.538%). This indicates that the real values obtained were in good agreement with the predictions of the quadratic equation model. Therefore, the model was considered suitable for predicting succinic acid production. Correspondingly, 74 g/L glucose, 30 g/L yeast extract, and 60 g/L $MgCO_3$ were obtained as the optimal points of the model. The optimal medium producing the actual maximum succinic acid was 60.09 g/L, and the predicted maximum of succinic acid was 59.89 g/L. The model obtained from BBD, and the RSM approach could be quite efficient and useful for the optimization of succinic acid fermentation by strain NP9-aA7.

### 3.8 Effects of Various Ratios of $Mg(OH)_2$ and $MgCO_3$

The mass ratio of $MgCO_3$ and $Mg(OH)_2$ at 3:1 gave the maximum 48.64 g/L succinic acid with a yield of 0.8132 g/g and cell growth of ($OD_{660}$) 17.61 (Table 3). Succinic acid gradually decreased with a decreasing proportion of $MgCO_3$ in the mixture. Only $MgCO_3$ (60 g/L) was used as a control medium when it produced a maximum of succinic acid of 41.523 g/L lower than 3:1

of $MgCO_3$ to $Mg(OH)_2$ (data not shown). Also, the $Mg^{2+}$ concentration in the mixed alkaline neutralizer (the ratio of $MgCO_3$ and $Mg(OH)_2$ was 3:1) was enough for the growth of cells and promote the succinic acid production. Moreover, the results of statistical analysis confirm that the optimal mass ratio for succinic acid production was 3:1 of $MgCO_3$ to $Mg(OH)_2$.

$Mg^{2+}$ derived from mixed alkaline neutralizers was sufficient to be used as a cofactor for the key enzyme in succinic acid fermentation. The complicity of mixed alkaline neutralizers (3:1 of $MgCO_3$ to $Mg(OH)_2$) could be increased solubility resulting in a homogeneous medium. It also supported cell growth and enhanced succinic acid concentration. From other research, using $Mg(OH)_2$ and NaOH mixture (1:1, w/w) by *A. succinogenes* NJ113 increased succinic production yield to 0.7260 g/g substrate [14].

### 3.9 Utilization of Sorghum Straw Hydrolysate (SSH) as a Carbon Source for Succinic Acid Fermentation

The cell growth of *A. succinogenes* NP9-aA7 and succinic acid production were examined when different concentrations of SSH were used as a carbon source. It was found to be able to grow in SSH in the range of 20-40 g/L. The maximum cell growth of ($OD_{660}$) was 4.615, and succinic acid was 19.14 g/L with a yield of 0.6323 g/g substrate at initial 40 g/L SSH. While a small level of growth was obtained when SSH was increased above 40 g/L, no succinic acid was detected (Table 3). The production of succinic acid in this research higher than previous research, Chen et al. [34] reported a succinic acid concentration of 15.50 g/L with a yield of 0.1240 g/g substrate was obtained from simultaneous saccharification and fermentation (SSF) of acid-pretreated rapeseed.

Recently, many researchers have reported lignocellulosic hydrolysates contain inhibitory compounds that affect cell growth and production of succinic acid.

For instance, Wang et al. [35] studied the inhibition effects of furfurals and 5-hydroxymethylfurfural (5-HMF) by recombinant *E. coli*. Cell growth and succinic acid production were entirely inhibited by furfural and HMF concentrations higher than 0.8000 g/L. While cell growth was completely inhibited either the concentration of furfural or HMF was over 6.400 g/L and 12.80 g/L, respectively. These inhibitors have effect on activity of key enzymes.

Lower succinic acid production from SSH as a carbon source may be the effect of the inhibiting components from the pretreated SSH such as HMF and furfural. However, it was still able to produce succinic acid. Therefore, further study should examine the inhibition and detoxification effects of inhibitors in the pretreated SSH to optimize conditions and improve the production process.

### 3.10 Succinic Acid Fermentation by *A. succinogenes* NP9-aA7 in a 2-L Fermenter

Based on the above results, batch fermentation was implemented in a 2-L stirred bioreactor. This result validated the model of optimal medium composition for succinic acid production from shaken flask cultivation. Correspondingly, 74 g/L glucose, 30 g/L yeast extract, and 60 g/L $MgCO_3$ were obtained as the optimal points of the model. The optimal medium producing the actual maximum succinic acid was 60.09 g/L, and the predicted maximum of succinic acid was 59.89 g/L. The model was thus validated in the fermentation resulting in the highest succinic acid concentration and yield at 27 h reaching 58.08 g/L with a yield of

0.8390 g/g glucose and maximum cell growth of 21.29 ($OD_{660}$).

There was no significance between the actual value and the predicted value (3.022%), and it indicates that the actual values obtained were in agreement with the predictions of the quadratic regression model. Therefore, the model was considered suitable for predicting succinic acid fermentation by *A. succinogenes* NP9-aA7. However, the succinic acid production could have occurred with less cultivation time than from the shaken flask scale, while the cell growth was higher. This result might be due to factors in the system throughout fermentation, for instance, aeration rate and pH controller in the culture which pH value was the key factor in cell growth and the production of succinic acid.

### 3.11 Effects of $CO_2$ Partial Pressure with $MgCO_3$

Figure 4 illustrates that when $CO_2$ was added in the fermentation resulting in the high amount of succinic acid. Using $CO_2$ at 25 kPa enhanced a maximum succinic acid of 53.46 g/L after 24 h of cultivation times. The lag phase of cell growth was observed for three h of cultivation time. The log phase of cell growth was in the range of 3.0-12 h, a maximum of cell growth at $OD_{660}$ of 18.88, growth rate of 0.3397 $h^{-1}$ then into stationary phase within 12-24 h. After that, the cell growth was slightly decreased to 7.560 ($OD_{660}$) at 48 h of cultivation times while the production of succinic acid appeared on the end of the stationary phase (24 h of cultivation times).The succinic acid was accumulated when the pressure of $CO_2$ was increased to 50.66 kPa. The maximum succinic acid of 72.93 g/L with a yield of 1.393 g/g glucose was obtained after 27 h of cultivation times. The succinic acid was increased by 36.44%. According to the maximum of cell growth ($OD_{660}$) was raised to 43.21 at 18 h of cultivation times and growth rate of 0.4758 $h^{-1}$. The increase of $CO_2$ affected the cell growth and succinic acid production. The more prolonged lag period was found in 75.99 kPa, and 101.3 kPa and succinic acid production were 59.24 and 65.85 g/L from 75.99 and 101.3 kPa of $CO_2$, respectively. At $CO_2$ 101.3 kPa, growth rate was reduced to 0.4494 $h^{-1}$.

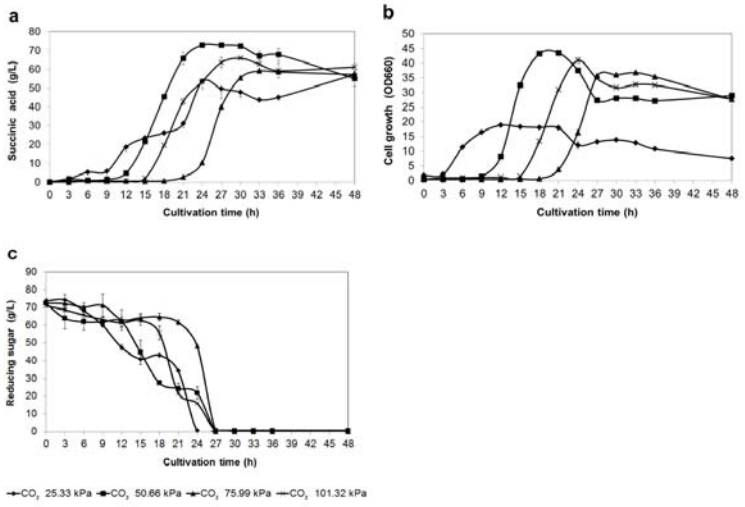


**Figure 4.** Effects of the supply of gaseous $CO_2$ on succinic acid production by *A. succinogenes* NP9-aA7. (a) The succinic acid production, (b) cell growth and (c) Residual sugars. Error bar is a standard deviation from triplicate experiments.

The result could be explained that the high level of dissolved $CO_2$ was favorable for the succinic acid production. Adding $MgCO_3$ also increased the concentration of dissolved $CO_2$. However, it was necessary to define the optimum condition of $CO_2$ and $MgCO_3$ for succinic acid production. In this research, gaseous $CO_2$ was used with $MgCO_3$ for control the pH resulting in more efficient on promoting the succinic acid production. Therefore, it was concluded that sufficient $CO_2$ at 50.66 kPa with 60 g/L alkaline neutralizer adding in the medium stimulated to produce succinic acid. Other similar research, Zou et al. [36] reported that adding 40 g/L $MgCO_3$ at the $CO_2$ partial pressure of 101.3 kPa enhanced the succinic acid to 61.92 g/L.

## 4. CONCLUSIONS

This research has found the optimal conditions for succinic acid production of *Actinobacillus succinogenes* NP9-aA7 (Accession No. LC192793). Using the combined statistical methods resulted in the development of higher succinic acid production. The maximum succinic acid of 60.09 g/L with a yield of 0.8161 g/g of glucose after 36 h in 50 ml flask fermentations. Moreover, The $CO_2$ of 50.66 kPa and alkaline neutralizers (45 g/L $MgCO_3$ and 15 g/L $Mg(OH)_2$) enhanced succinic acid to 72.93 g/L with a yield of 1.393 g/g glucose after 24 h in a 2-L fermenter. It might be beneficial for bio-based succinic acid in industries for various applications.

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