

Optimization of Slurry Fermentation for Succinic Acid Production by Fungal Co-Culture

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This study attempted to produce succinic acid from co-culture of *Aspergillus niger*, *Trichoderma reesei*, and *Phanerochaete chrysosporium* strains in a two-step fermentation: solid-state fermentation pre-culturing phase followed by slurry fermentation. Response surface methodology (RSM) via central composite design (CCD) was used to optimize and investigate the effects of slurry fermentation variables such as initial acetic acid concentration, initial pH, and solids loading on succinic acid yield. These fermentation variables were identified as key factors influencing succinic acid yield based on preliminary studies. Experimental validation of the optimum conditions (4.58 g/L of acetic acid, initial pH of 4.69, and 12.92% (w/w) of solids loading) produced 32.58 ± 0.18 g succinic acid per kilogram dry substrate, which agreed with the predicted yield of 32.25 g succinic acid per kilogram dry substrate. The interaction between the acetic acid and initial pH possibly affected the permeability of acetate and succinate anions to the cells resulting in the accumulation of extracellular succinic acid in the slurry. High solids loading was observed to limit mixing and mass transfer of the slurry components. The results of this study will be useful in performing kinetic studies to deepen the understanding of producing succinic acid using fungal co-culture.

1. Introduction

In recent years, bio-based chemicals are gaining interest due to their promising potential. Succinic acid, a 1,4 butanedioic acid is one of them. It has a variety of uses whether in direct applications in food, pharmaceuticals, surfactants, and green solvents, or as a raw material in synthesizing various value-added chemicals (Zeikus et al., 1999). Traditionally, succinic acid is produced from petroleum products. However, interest in producing succinic acid through a more sustainable route, i.e., microbial fermentation by some rumen bacteria that are natural producers of succinic acids or by modified microorganisms specially designed to produce succinic acid (Song and Lee, 2006). This study explored another alternative route in producing succinic acid by utilizing lignocellulosic biomass as feedstock and by harnessing the synergistic capability of fungi to simultaneously produce enzymes that will degrade the biomass into simple sugars and ferment these sugars to succinic acid. Preliminary studies conducted by Alcantara et al. (2017) demonstrated these through a two-step fermentation process — solid-state fermentation pre-culturing phase, where lignocellulolytic enzymes were produced, followed by slurry fermentation phase, where simultaneous enzymatic hydrolysis and fermentation happened. Alcantara and Mondala (2020) also identified the important role of acetic acid to induce succinic acid production of the fungal co-culture of *Aspergillus niger*, *Trichoderma reesei*, and *Phanerochaete chrysosporium* in a two-step fermentation process.

In this study, only the slurry fermentation phase was optimized as there are several studies on that optimized variables in solid-state fermentation by cellulolytic enzymes (Latifian et al., 2007; Mekala et al., 2008; Brijwani et al., 2010). Statistics-based optimization of batch fermentation variables such as medium components and/or operational parameters has also been employed to maximize succinic acid production of succinic acid-producing bacteria such as modified *Escherichia coli* (Isar et al., 2006) and *Actinobacillus succinogenes* (Borges and Pereira, 2011).

To the knowledge of the authors, optimization of slurry fermentation variables for succinic acid production from the direct utilization of lignocellulosic biomass using fungal co-culture has not been done by other studies.

The objectives of this study were to optimize the slurry fermentation parameters such as initial acetic acid concentration, initial pH, and solids loading for maximal succinic acid yield and to determine the effects of these variables on succinic acid yield.

2. Materials and Methods

2.1 Substrate, medium, and inoculum preparation

Birchwood chips (BWC) and soybean hull (SBH) were the substrates used in this study. Both substrates were sequentially air-dried, milled, screened to a particle size range of 500 to 1,000 μm , and stored separately. Mandel's medium (Mandel and Weber, 1969) with modification by Tangnu et al. (1981) was prepared by dissolving 1.4 g $(\text{NH}_4)_2\text{SO}_4$, 2.0 g KH_2PO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g $\text{CaCl}_0 \cdot 2\text{H}_2\text{O}$, 0.3 g NH_2CONH_2 , 1.0 g Proteose Peptone No. 2, 0.2 mL Tween 80, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 mg CoCl_2 in 1 L deionized water. The fungal strains used were *Aspergillus niger* Y-78 (ATCC 15475), *Phanerochaete chrysosporium* A-381 (ATCC 48746), and *Trichoderma reesei* RUT-C30 (ATCC 56765). The fully sporulated spores from slants of the three fungal strains were separately suspended using sterilized basal medium, collected, and poured into potato dextrose agar (PDA) plates. The plates were incubated for seven days at 25 °C. Then the spores were collected and transferred to a 250 mL Erlenmeyer flask. The spore loading was adjusted to 1×10^7 spore/mL by diluting it with sterilized basal medium with 2% w/v glucose supplementation. The spore suspensions were incubated in a shaker (Innova® 43, New Brunswick Scientific Co. Inc., NJ, USA) at 30 °C and 150 rpm for two days.

2.2 Solid-state fermentation pre-culturing

5 g and 15 g of milled SBH and BWC, respectively were separately put in 250-mL Erlenmeyer flasks. The moisture content was adjusted to 70% (w/w) using the basal medium and then autoclaved at 121 °C for 15 min and allowed to cool to room temperature. The flask containing SBH was inoculated with 5 mL of the two-day old *T. reesei* culture. After 24 hrs, 5 mL of the two-day old *A. niger* culture was inoculated to the same flask containing the SBH substrate and the *T. reesei* culture. The flask containing BWC was inoculated with 5-mL of the two-day *P. chrysosporium*. Both the inoculated SBH and BWC flasks were kept at 30 °C and 95% relative humidity in a humidifier incubator (HIS33SD, Powers Scientific Inc., PA, USA) for 7 days.

2.3 Slurry fermentation

The 7-day old *A. niger-T. reesei* culture on SBH and *P. chrysosporium* culture on BWC were combined in a 250 mL Erlenmeyer flask using sterilized acetate buffer to transfer all solids in both solid-state pre-cultures. The amount of initial acetic acid concentration, initial pH, and solids loading were varied. All experimental runs were covered with foam stopper and placed in an incubator shaker (Innova® 43, New Brunswick Scientific Co. Inc., NJ, USA) and were initially agitated at 250 rpm for 20 minutes at 30 °C to thoroughly mix the combined cultures. Then the agitation speed was lowered to 150 rpm and the resulting slurries were incubated for the next 5 days at 30 °C.

2.4 Analytical method

Samples (1.0 mL) were collected from the liquid portion of the broth and transferred to a 1.5-mL Eppendorf tube. The tubes were centrifuged in a microcentrifuge (5415D, Brinkman Instrument Inc., NY, USA) at 12,000 rpm and 10 minutes. The supernatants were collected and diluted to 1:3 with deionized water. The diluted samples were analyzed for succinic acid content using Agilent 1100 Series HPLC (Agilent Technologies, CA, USA) equipped with BioRad HPX-87H Cation Exchange column and Variable Wave Detector (VWD) at 30 °C, 0.005 M H_2SO_4 mobile phase, UV absorbance at 210 nm, at a flow rate of 0.5 mL/min. The standard used was an analytical grade succinic acid (Sigma Aldrich, MO, USA).

2.5 Experimental design

Response surface methodology was used to optimize slurry fermentation variables for maximum succinic acid yield and to investigate the effects of these fermentation variables in succinic acid production of the fungal co-culture. Initial acetic acid concentration (X_1), initial pH (X_2), and solids loading (X_3) were identified as independent slurry fermentation variables from preliminary studies. Central composite design was applied to generate experimental runs. A total of 20 experimental runs with 6 repeated runs at central levels were generated. The axial-low, low, middle, high, and axial-high levels are summarized in Table 1. Succinic acid yield in g succinic acid per kg dry substrate was the dependent output of the study. Statistical analysis was performed using Minitab® 18 software (Minitab Inc., PA, USA).

Table 1: Actual levels of slurry fermentation variables

Variables	Coding	Coded Levels				
		- α	-1	0	1	+ α
Acetic acid concentration, g/L	X_1	1.65	2.50	3.75	4.00	5.85
Initial pH	X_2	4.30	4.50	4.80	5.10	5.30
Solids loading, % (w/w)	X_3	6.59	10.00	15.00	20.00	23.41

3. Results and Discussion

3.1 Response surface optimization and verification

The significant independent variables in the succinic acid production by the fungal co-culture in slurry fermentation, namely initial acetic acid concentration (X_1 , g/L), initial pH (X_2), and solids loading (X_3), %, w/w), were identified based on preliminary studies. Central composite design matrix with corresponding experimental results is presented in Table 2. Twenty experimental runs were conducted at different combination of variable levels and the responses were measured in terms of succinic acid yield. The highest succinic acid yield of 31.76 g/kg was observed at one of the centre levels where initial acetic acid concentration, initial pH, and solids loading are 3.75 g/L, pH 4.80, and 15% (w/w), respectively. On the other hand, no succinic acid was observed in runs 3 and 9 where initial acetic acid concentration, initial pH, and solids are 2.50 g/L, pH 5.10, and 10% (w/w), and 1.65 g/L, pH 4.80, and 15% (w/w), respectively, after 5 days of slurry fermentation.

Table 2: Central composite design matrix with experimental succinic acid yield

Runs	Variables			Succinic Acid Yield (g/kg)
	Initial Acetic Acid Concentration (g/L) X_1	Initial pH X_2	Solids Loading (% w/w) X_3	
1	2.50	4.50	10.00	25.72
2	5.00	4.50	10.00	26.30
3	2.50	5.10	10.00	0.00
4	5.00	5.10	10.00	29.14
5	2.50	4.50	20.00	24.95
6	5.00	4.50	20.00	28.02
7	2.50	5.10	20.00	2.13
8	5.00	5.10	20.00	25.42
9	1.65	4.80	15.00	0.00
10	5.85	4.80	15.00	28.70
11	3.75	4.30	15.00	28.57
12	3.75	5.30	15.00	2.09
13	3.75	4.80	6.59	26.33
14	3.75	4.80	23.41	14.87
15	3.75	4.80	15.00	31.76
16	3.75	4.80	15.00	25.33
17	3.75	4.80	15.00	25.79
18	3.75	4.80	15.00	29.87
19	3.75	4.80	15.00	28.09
20	3.75	4.80	15.00	29.00

The observed responses were fitted in a second order polynomial equation giving succinic acid yield as a function of initial acetic acid concentration, initial pH, and solids loading. The quadratic model generated, shown in Equation 1, includes the main effects, quadratic effects, and two-factor interaction effects. To improve the quadratic model, insignificant model terms were eliminated using backward elimination method. Both the interaction between initial acetic acid concentration and solids loading, X_1X_3 and the interaction between the initial pH and solids loading, X_2X_3 were statistically insignificant; thus, these were eliminated from the quadratic model. By default, Minitab[®] 18 uses a confidence level of 90% when performing backward elimination of insignificant model terms. ANOVA was conducted to test the validity of the regression model. The model is significant since its p-value is less than 0.10. The lack-of-fit is not significant relative to the pure error since its p-value is greater than 0.10, which means that the experimental response fits the model well.

The model determination coefficient, R^2 of 0.9348, was in reasonable agreement with the experimental response which means that the model could explain 93.48% of the variability in the response. In addition, the adjusted R^2 of 0.8968 was in reasonable agreement with the predicted R^2 of 0.7273. Thus, the quadratic model is a good predictive tool to guess the succinic acid yield within the constraint of the independent variables.

$$y = -592 + 52.5X_1 + 312X_2 + 1.93X_3 - 2.598X_1^2 - 41.2X_2^2 - 0.0739X_3^2 + 16.26X_1X_2 \quad (1)$$

The regression model generated response surface plots, shown in Figure 1, to show the interaction among different variables within their experimental ranges with one factor held constant at the middle level. The 3-D plots are curved due to the presence of significant quadratic terms in the regression model. The response surface plots were useful in observing the response behaviour of succinic acid yield at different variable levels and can be used to estimate the best combination of slurry fermentation variables that can give the highest succinic acid yield.

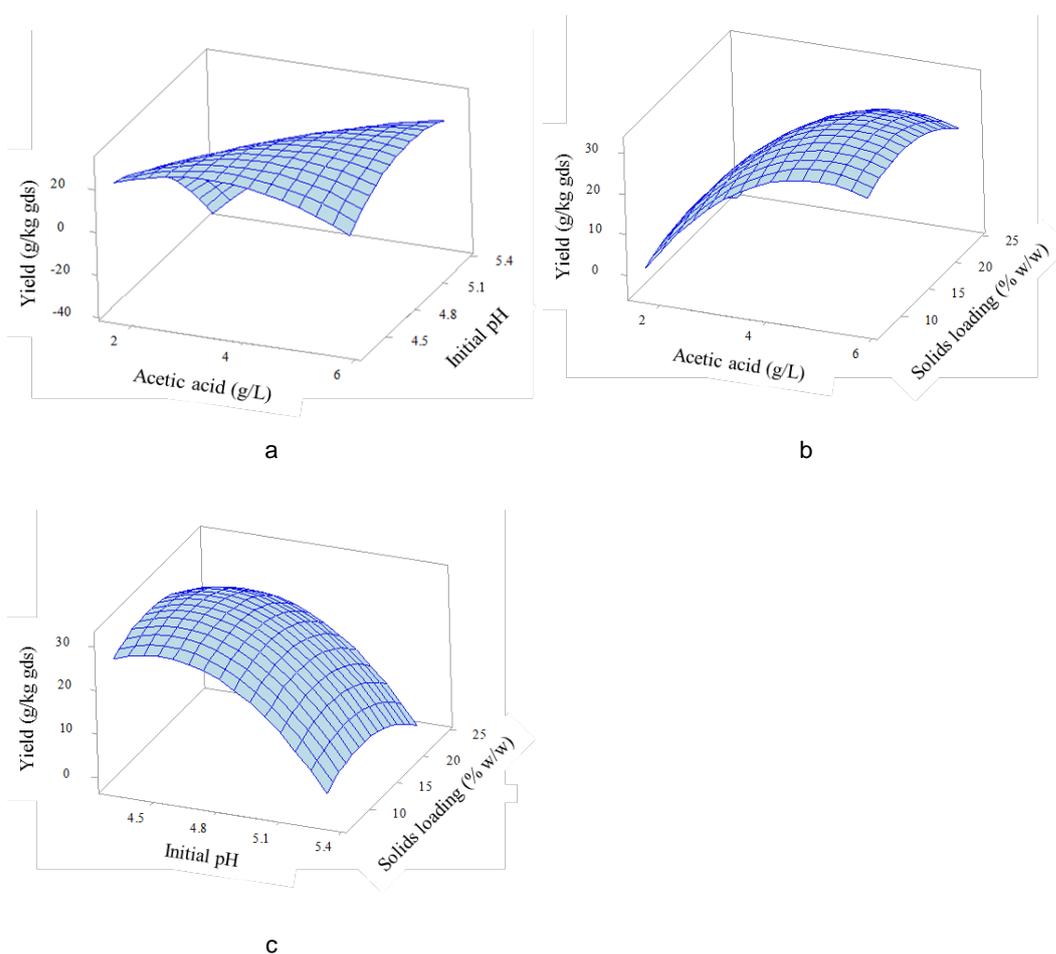


Figure 1: Response surface plots showing the effects of a) initial acetic acid concentration and initial pH, X_1X_2 , b) initial acetic acid concentration and solids loading, X_1X_3 , and c) initial pH and solids loading, X_2X_3 on succinic acid yield.

The optimum levels of the slurry fermentation variables were determined using Minitab[®] 18's response optimizer. The variables were constrained within their experimental limit. The solution with the highest desirability (desirability = 1) was chosen as the optimum conditions. The optimum initial succinic acid concentration, initial pH, and solids loading were 4.58 g/L, pH 4.69, and 12.92% (w/w), respectively, with a predicted response of 32.25 g succinic acid/kg dry substrate. The quadratic model was experimentally validated using the recommended optimum conditions.

The observed succinic acid yield of 32.58 ± 0.18 g succinic acid/kg dry substrate. The experimental succinic acid yield was within 95% confidence interval of the predicted response. Thus, the quadratic model was reasonably reliable and accurate in predicting succinic acid yield from slurry fermentation using fungal co-culture.

3.2 Effect of slurry fermentation variables

The strength of acetic acid in the slurry is considered one of the most crucial factors in succinic acid production. Acetic acid has an inhibiting effect on succinate anion uptake of *A. niger* (Fencel and Leopold (1958)). The reported inhibition concentration of undissociated acetic acid for succinate anion uptake was above 2.5 mM which is equivalent to 1.5 g/L acetic acid in the slurry. No succinic acid was produced in the run with less than 1.5 g/L of acetic acid. The center point in this study was set to 2.5 g/L which is above the inhibiting concentration of acetic acid. The high and axial high levels considered in this study did not inhibit succinic acid production as these levels were below the growth-inhibiting concentration of acetic acid on *Aspergillus* species. Hassan et al. (2015) reported this acetic acid concentration at 5% (52.2 g/L). The high values considered in this study were lower than the growth-inhibitory concentration of acetic acid.

Results of preliminary experiments of succinic acid production from slurry fermentation by fungal co-culture favored a particular pH range. With this, initial pH was considered as one of the slurry fermentation variables. The middle level was set at pH 4.80, similar to the pH used for filter paper assay (Ghose, 1987) for optimum cellulase hydrolysis. Li et al. (2013) identified the optimal pH range of 4.5 to 5.0 for batch fermentation process without pH control. Control of pH level was not employed during the 5-day slurry fermentation phase. Lignocellulosic enzymes were primarily produced during the solid-state pre-culturing phase. The introduction of mixing during the slurry fermentation phase increased the contact between the enzymes and the substrates to liberate fermentable sugars. In addition, the synergistic interaction of these enzymes would hydrolyze the lignocellulosic biomass components to fermentable sugars to be readily available for the fungi, specifically *A. niger*, to produce succinic acids in the slurry. In addition, the pKa of acetic acid is 4.75 where both the undissociated acetic acid and acetate anions are present in equilibrium. The pH value outside the optimal pH range could decrease enzymatic activities or even deactivate the enzymes leading to low yield of fermentable sugars that can be converted to succinic acid.

Solids loading can significantly alter the physical properties of the slurry. At low level solids loading, the slurry appeared to be diluted due to high ratio of liquid present in the slurry. Results showed that low level of solids loading did little effect on succinic acid yield. Over-dilution of the slurry can potentially pose a problem in the downstream process of recovering succinic acid from the solution. In contrast, high solids loading slightly resembles that of solid-state fermentation due to the reduced amount of liquid present. High solids loading resulted to difficulty in mixing that hinders mass transfer in the process. Poor mixing resulted to lower succinic acid yield as there can be a decreased contact between the enzymes and substrates to produce fermentable sugars available to be converted to succinic acid.

4. Conclusions

This study performed a two-step fermentation process — solid-state pre-culturing phase followed by slurry fermentation — to produce succinic acid from lignocellulosic biomass using fungal co-culture. In the solid-state pre-culturing phase, *A. Niger*, *T. reesei*, and *P. chrysosporium* were initially grown on moist substrate to produce lignocellulolytic enzymes. In the slurry fermentation phase, simultaneous enzymatic hydrolysis and succinic acid fermentation occurred. The effects of initial acetic acid concentration, initial pH, and solids loading were determined and optimized using central composite design and response surface methodology for maximum succinic yield. This method optimizes the independent variables to yield the maximum succinic acid by fitting the experimental data in a quadratic model. The validity of the model was tested using a series of statistical analyses. Results showed that all quadratic effects of the three variables and the interaction of initial acetic acid and initial pH were statistically significant in the regression model. To produce the maximum succinic acid yield of 32.58 ± 0.18 g succinic acid/kg dry substrate, slurry fermentation should contain 4.58 g/L acetic acid, pH 4.69, and 12.92% (w/w) solids loading. The optimum conditions were also experimentally verified and showed that the experimental yield is in close agreement with the predicted succinic yield of 32.25 g succinic acid/kg dry substrate. When the initial acetic acid concentration is more than 1.5 g/L, accumulation of succinic acid was observed in the broth. The initial pH also played a significant role in sustaining high enzymatic activity and in converting the lignocellulosic biomass to fermentable sugars. Solids loading affected the ease of mixing to maximize enzymatic activity and reducing sugar uptake of the fungal co-culture.

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