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Modelling $MgCO_3$ Buffered Succinic Acid Production by *Actinobacillus Succinogenes*

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Thie current study focused on using a previously published model known to accurately predict the cultivation of *Actinobacillus succionogenes* on glucose in a NaOH neutralised system, for the prediction of batch system behaviour when $MgCO_3$ is used as a pH buffer in a batch reactor system. The use of $MgCO_3$ as a neutralising agent resulted in a biomass growth lag of about 8 h, followed by a sharp increase untill 15 h at which point the biomass concentration remained constant throughout the fermentation run. The maximum biomass concentration was 3.62 g/L. The fermentation ceased at a final SA concentration of 38.12 g/L and the SA yield on glucose was 0.81 g/g from an initial glucose concentration of 60 g/L. At the end of the fermentation, 79 % of the glucose as consumed. A residual glucose concentration of 12.84 g/L at the maximum SA concentration indicated product-related inhibition. The biomass, acetic acid (AA), and formic acid (FA) concentrations were incorrectly predicted by the model which implies that the use of $MgCO_3$ as a buffer agent changed the metabolic fluxes of the organism.

1. Introduction

Succinic acid (SA) is a natural organic acid that exists in plant and animal tissue (Yang et al., 2019) because it plays a significant role in their intermediatory metabolism (Nghiem et al., 2017). It has been identified as one of the top 10 potential value-added chemicals from biomass by the US Department of Energy due to its potential as a platform chemical which has a wide range of applications in industries such as agriculture, food, metal, chemical and pharmaceutical as a precursor, ion chelator and/or additive agent (Putri et al., 2020). One major development of succinic acid is its use in the production of many specialised polyesters such as polyester polybutylene succinate (Cao et al., 2013).

The highest production of bio-succinic acid is achieved by anaerobic microbial fermentation (Brink and Nicol, 2014; Putri et al., 2020). *Actinobacillus succinogenes* is recognized as one of the most promising succinic acid producers (Dessie et al., 2018) because of its ability to metabolise a wide range of carbon sources (Ferone et al., 2019) to produce SA at considerable high titres, productivities, and yields (Bradfield and Nicol, 2016; Mokwatlo et al., 2020), partly due to its high tolerance of glucose and SA concentrations. *A. succinogenes* has the ability to self-immobilize and form biofilms during continuous operation (Brink and Nicol, 2014; Maharaj et al., 2014; Bradfield et al., 2015). The formation of biofilms increases cell densities in the bioreactor which in turn increases the SA productivities (Mokwatlo et al., 2021).

One of the critical factors that affect bio-SA production is the pH of the fermentation medium. It influences the cellular metabolism of the microbe (Wang et al., 2012, Putri et al., 2020). The optimal pH for *A. succinogenes* growth is 7.0 *(Dessie et al., 2018).* Therefore, the inclusion of pH regulators in the fermentation medium is important because the production of organic acids such as acetic acid, SA and formic acid during the fermentation process will acidify the medium. Many publications have named $MgCO_3$ as the most effective pH control agent for *A. succinogenes* because it supplies CO_2 and Mg^{2+} ions that serve as co-factors for many enzymes in the SA synthesis pathway (Wang et al., 2011; Dessie et al., 2018). Yu et al. (2010) also found that $MgCO_3$ gave a higher SA production and less by-product formation. Most batch fermentation studies have

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demonstrated a decrease in biomass concentration soon after the maximum growth has been reached (Wang et al., 2012; Salvachu et al., 2016; Lexow et al., 2021). Wang et al. (2012) and Lui et al, 2010 reported a lag phase in the biomass growth when the fermentation medium was supplemented with $MgCO_3$.
Due to the importance of SA in industry, many studies have developed models to simulate, control and optimize the bio-SA production process (Pateraki et al., 2016) but most kinetic studies are based on batch fermentation. Most batch studies indicate that SA production is unaffected after the growth termination point (Brink and Nicol, 2014). Corona-González et al. (2008) reported a model that describes both product and substrate inhibition effects on SA production from glucose by *A. succinogenes* during batch fermentation. Mokwatlo et al. (2021) developed a biofilm model using intrinsic SA production kinetics estimated from resuspended batch biofilm fermentation experiments.
The current study will seek to validate use of $MgCO_3$ as an effective neutralizing agent for SA production. The kinetics proposed by Mokwatlo et al. (2021), will be used to predict the SA production by including the substrate and product inhibition in the specific growth model.

2. Materials and methods

2.1 Microorganism and growth medium

Actinobacillus succinogenes 130Z (DSM No. 22257; ATCC No. 55618) was acquired from the German Collections of Microorganisms and Cell Cultures (Braunschweig, Germany). Stock cultures (1.5 mL) are stored at − 40 °C in 66 % v/v glycerol solutions. Inoculum was prepared by transferring a stock culture to 15 mL of sterilised tryptone soy broth at 30 g/L and incubating at 37 °C and 150 rpm for 16 to 24 h. Prior to inoculation a High-performance liquid chromatography (HPLC) was used to analyse the inoculum for purity by checking for consistent metabolite distribution.

2.2 Fermentation media

The fermentation medium was a replica of the medium developed by Bradfield and Nicol (2014). All chemicals were obtained from Merck KGaA (Darmstadt, Germany) unless otherwise stated. The medium consists of three parts: the nutrient and salt solution, a phosphate buffer, and the glucose/substrate solution. The nutrient and salts solution were composed of 6.0 g/L yeast extract, 10.0 g/L clarified corn steep liquor (Sigma-Aldrich, St Louis, USA), 0.5 g/L NaCl, 0.2 $MgCl_2{\cdot}6H_2O$, 0.2 g/L $CaCl_2{\cdot}2H_2O$ and 1.0 mL/L of Antifoam SE-15 (Sigma-Aldrich, Germany). The phosphate buffer consisted of 1.6 g/L KH_2PO_4 and 0.8 g/L K_2HPO_4. The D-glucose (Futaste Pharmaceutical Co. Ltd, Shandong, China) concentration will be maintained at 66 g/L. $MgCO_3$ acted as the pH regulator and the final concentration in the fermenter was 40 g/L.

2.3 Experimental setup and operation

The three feed solutions will be prepared in different bottles and were diluted with distilled water until a 250 mL solution was obtained. The initial fermenter broth volume was 750 mL. All three bottles were that were connected in series with silicone tubing to a 1 L fermenter. The silicone tubing was clamped shut to prevent mixing and unwanted reactions from occurring during autoclaving and were separately sterilised by autoclaving at 121°C for 60 min. Once they have cooled down, three solutions were added to the main reactor, aseptically.
The fermenter was placed on a hotplate stirrer and attached to a temperature measuring electrode. Temperature and stirring speed were controlled at a temperature of 37 °C and 500 rpm, respectively. Inoculum was added into the reactor under sterile conditions and anaerobic batch fermentations were carried out for a total of 48 hours. A sample was taken from the onset of the batch fermentation to note initial conditions for a batch run. Samples are taken every 2 hours for the first 12 hours and then the sampling time is increased.

2.4 Analytical methods

Each sample was diluted with 3 mL of a 1 mol/L HCl solution to break down the carbonate solids that form inside the reactor.
Concentrations of Glucose (Glc), ethanol, and organic acids – succinic acid (SA), acetic acid (AA) and formic acid (FA) – in the fermenter broth were determined by High-Performance Liquid Chromatography (HPLC). An Agilent 1260 Infinity HPLC (Agilent Technologies, USA), equipped with an RI detector and a 300 mm × 7.8 mm Aminex HPX-87H ion exchange column (Bio-Rad Laboratories, USA) was used. Two mobile phases were used for two methods of analysis. The first method consisted of a 5 mM H2SO4 mobile phase solution fed at a flowrate of 0.6 mL min^{-1} and the second method used a 20 mM H2SO4 mobile phase at the same

flowrate. The second method improved the accuracy of the glucose reading by separating the phosphate, glucose, and pyruvic acid peaks.
The biomass concentration (X) in the reactor was approximated by performing absorbance readings using a T60 UV-Visible Spectrophotometer (PG Instruments Limited, UK) at a wavelength of 600 nm.

2.5 Mathematical modelling

The mathematical equations used to model this batch reactor system were previously published by Mokwatlo et al. (2021). Although the system used in that study was a biofilm batch reactor which was initially operated as a pseudo-steady state continuous operation. The specific growth rate of *A. succinogenes* was modelled using the product and substrate inhibition kinetics reported in (Brink and Nicol, 2014) shown in Eq (1). The maximum specific growth of 0.82/h, SA (C_{SA}) and Glc concentration (C_{Glc}) as the product and substrate inhibition variables, respectively. The growth (Φ) and maintenance (ɵ) production rates were used to simulate the cell-based production rate of SA (r'_{SA}) in Eq (2) and (3) which is adopted from (Mokwatlo et al, 2021).

$$\mu = 0.82\left(1 - e^{-k_1 \times \left(e^{-k_2 C_{SA}}\right)}\right) \times \left(\frac{C_{Glc}}{K_S + C_{Glc}}\right) \quad (1)$$

$$r'_{SA} = \Phi\mu + \theta \quad (2)$$

$$\theta = \frac{kC_{SA}}{K_P + C_{SA} + \left(\frac{{C_{SA}}^2}{K_I}\right)} \quad (3)$$

The mass ratio functions in Eq (4) and (5) are used to relate the SA production rate the AA and FA production rates. Eq (6) is used to model the glucose consumption rate. Table 1 shows the model parameters found in Mokwatlo et al. (2021).

$$r'_{AA} = \left(1.112{C_{SA}}^{-0.445}\right) r'_{SA} \quad (4)$$

$$r'_{FA} = \left(0.6214e^{-0.09C_{SA}}\right) r'_{SA} \quad (5)$$

$$r'_{Glc} = r'_{sA} \times \left(0.89 + Y_{\frac{AA}{SA}} + 0.33Y_{\frac{FA}{SA}}\right) \quad (6)$$

Table 1: Model parameters for Eq (2) and (3)

Symbol	Values	Units
Φ	3.3	g/g
k	3	g/g.h
K_P	13.15	g/L
K_I	7.52	g/L
K_S	3	g/L

3. Results and discussion

The growth rate profile is characterized by a lag phase of approximately 8 h, which is followed by a sharp increase until it stops at 15 h and remains constant at a maximum biomass concentration of 3.62 g/L until the end of the fermentation time (Figure 1). This proves that biofilm formation was suppressed with the use of $MgCO_3$ as a pH buffer. Consequently, the model does not accurately model the biomass growth rate, and this could be due to the difference in pH buffer used to create the model used by Brink and Nicol (2014) and

Mokwatlo et al. (2021). The publications that have used $MgCO_3$ as a buffer have reported an initial biomass growth lag (Liu et al., 2010; Wang et al., 2012).
Although the growth rate remains constant, the succinic acid production continues to increase as shown in Figure 2, which implies that more carbon is being used for SA production instead of growth. The maximum SA produced was 38.12 g/L.

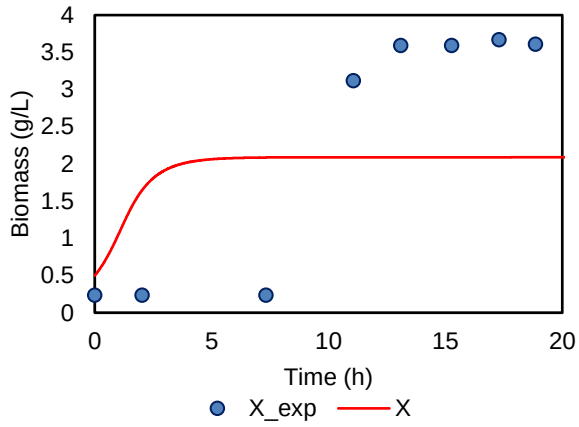

Figure 1: Bacterial growth in glucose by A. succinogenes. The figure shows that the model significantly under-predicted the growth rate.

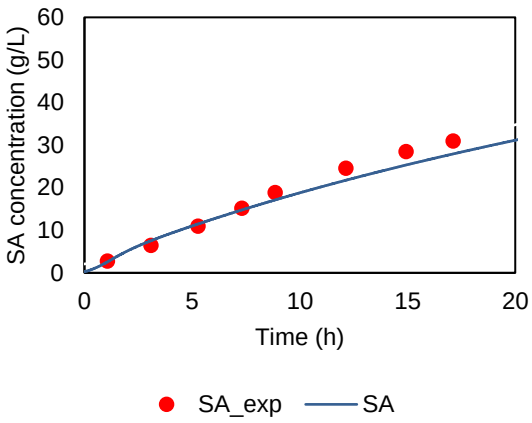

Figure 2: Succinic acid production profile on pure glucose by A. succinogenes.

When the lag time is considered, the SA production rate and the glucose consumption rate are predicted well as seen in Figure 2 and Figure 3, respectively. This shows that the flux of SA from Glc remains unchanged for the $MgCO_3$ buffer as compared to NaOH neutralization. Table 2 reports the initial and final concentrations of the glucose and organic acids during the batch fermentation. The succinic acid yield on glucose was 0.81 g/g-close to the theoretical redox neutral limit of 0.87 g/g (Bradfield and Nicol, 2014). At 40 h, 79% of the substrate was consumed. Salvachúa et al. (2016) reported that 100 % of the glucose substrate was used up at 40 h but this could be due to the Na_2CO_3 pH buffer used in that experiment. A residual glucose concentration of 12.84 g/L at the maximum SA concentration indicated product-related inhibition. The acetic and formic acid concentrations in Figure 4 were not accurately modelled by these equations. The model predicted that these by-product acids that would form would be double what was experimentally achieved. This proves that $MgCO_3$ does reduce the amount of by-product acid formation as reported by Yu et al. (2010).

Table 2: Summary of the initial and final experimental concentrations of Glc, SA, AA, and FA

Substrate/Product	Initial concentration (g/L)	Final Concentration (g/L)
Glucose	60.0	12.84
SA	0.208	38.12
AA	0.0513	8.00
FA	0	3.79
X	0.23	3.62

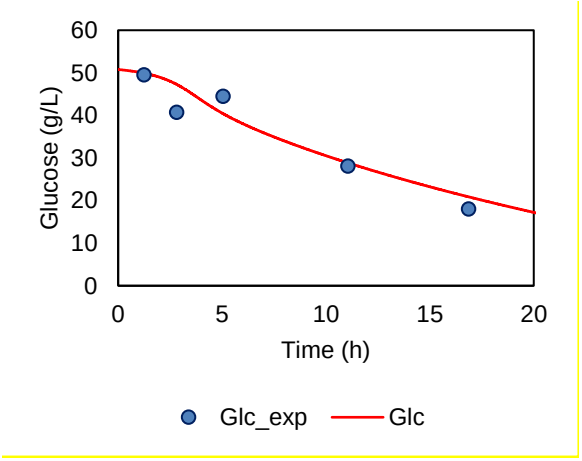

Figure 3: Sugar consumption by A. succinogenes on pure glucose.

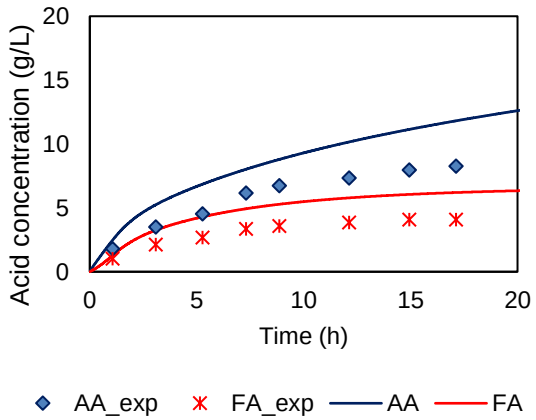

Figure 4: Acetic acid and formic acid concentration on glucose by A. succinogenes on glucose.

4. Conclusions

In this study, the Mokwatlo et al (2021) model to predict SA production kinetics was tested to determine its validity in a batch system utilising $MgCO_3$ as neutralizing agent. It should be noted that $MgCO_3$ suppressed biofilm formation without affecting the SA production rate as the maximum biomass concentration remained constant throughout the fermentation time. $MgCO_3$ was however effective in reducing the amount of by-product acid production rate, resulting in underprediction of the by-product formations by the model. In addition, the model did not consider the initial biomass growth lag caused by this buffer, and it significantly underestimated the maximum biomass concentration. However, the model was able to accurately predict the succinic acid and glucose titres demonstrating its potential of the model as a screening tool to predict A. succinogenes behaviour under batch fermentation conditions.

Nomenclature

Glc – Glucose
SA – Succinic Acid
AA – Acetic Acid
FA – Formic Acid
X – Active biomass
k – Maximum maintenance associated production rate, g/(g/h)
K_P – Monod constant in maintenance associated production rate, g/L
K_I – Inhibition constant in the maintenance associated production rate, g/L
Ks – Substrate inhibition constant on specific growth rate, g/L
μ – Specific growth rate (h^{-1})
ɵ - Maintenance associated production rate coefficient, g/(g.h)
Φ – Growth associated production rate coefficient, g/g

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