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THE EFFECTS OF REDUCING POWER FROM METAL CARBONATES ON SUCCINIC ACID PRODUCTION USING *ACTINOBACILLUS SUCCINOGENES*

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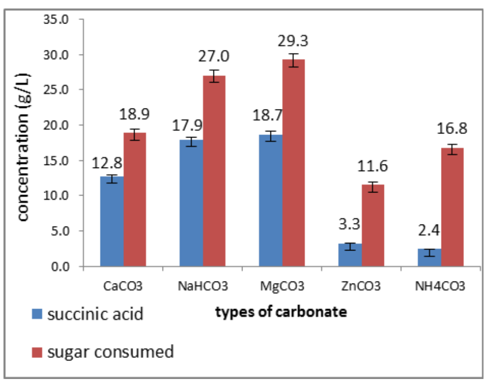
Graphical abstract



Abstract

Bio-succinic acid was first commercialized in 2012 and has a potential to hit $ 15 billion market. Higher conversion or yield in this case, allow bio-succinic acid to be more competitive and could potentially take over the market of other intermediate compound. Redox state of the fermentation medium is reported to have an effect on the metabolic flux and in *Actinobacillus succinogenes*, one of the best succinic acid producers. It has a direct impact on succinate yield. Therefore, carbonates with different reducing power, $CaCO_3$, $NaHCO_3$, $MgCO_3$, $ZnCO_3$ and $(NH_4)_2CO_3$ were employed to investigate the effect of their reducing power on succinate yield from glucose. Results showed that a more reducing carbonate, $CaCO_3$ promoted the metabolic flux of *A. succinogenes* to produce higher reductive metabolite (succinate) to regain its intracellular redox balance. Calcium has a reducing power of 2.87 relative to hydrogen. Ammonium carbonate has an oxidizing power of 0.27, or a negative reducing power relative to hydrogen. Therefore, $CaCO_3$ with a stronger reducing power had a higher succinate yield of 0.68 g/g than $(NH_4)_2CO_3$ with only 0.14 g/g. On the other hand, $MgCO_3$ had the highest final succinate concentration due to the overall higher sugar consumption by *A. succinogenes*.

Keywords: Bio-succinic acid, reducing power, succinate yield, metabolic fluxes, fermentation

1.0 INTRODUCTION

Over the last decade, the rising concern on the environmental impact and sustainability issues has spurred bio-economy growth. As a result, quite a good number of chemical building blocks have been commercialized by means of biological route. In 2004, US Department of Energy had listed 12 potential chemical building blocks to be commercialized and succinate was the first in the list. Succinate is typically produced via petrochemical platform from the catalytic hydrogenation process of malate [3]. However, commercial bio-production of succinate has already been realized and as of 2014, about 12 commercial bio-succinate production plants are in operating stage around the globe [4]. Apart from reducing 50 % of the greenhouse gas emissions, life-cycle analysis of bio-succinic acid showed that, it lowers the energy consumption by 30−40 % as compared to petrochemical method [3].

The current market volume of succinic acid, reported by the Royal Society of Chemistry in 2014, is around 30,000 tonnes per annum, creating a market capacity of $225 million. The world succinic acid market is forecast to grow at an 18.7% compound annual growth rate (CAGR) from 2011 to 20168. Succinate can achieve a $15 billion market by producing bulk chemicals such as ethylenediamine disuccinate (a biodegradable chelator), diethyl succinate (a replacement of methylene chloride), 1,4-butanediol (a plastic precursor) and adipic acid (nylon precursor) [5], it has a potential $15 billion worth of market [6]. The early players of bio-succinic acid include Reverdia, Purac, BASF, BioAmber and several others [4].

In order to promote bio-succinic acid industry, production cost has to be made competitive with the maleic anhydride industry. Maleic anhydride is in increasing demand in spite of rising oil prices [7]. The economic viability of a bio-process is governed by three important process parameters: titer, yield, and productivity [8; 9]. Yield or the conversion of raw material into product succinic acid affects significantly on the cost of the raw feedstock per kg of succinic acid produced which is now in increasing importance due to the rising price of sugar. Therefore, several studies have been conducted to improve the efficiency of the bio-production by enhancing the yield of succinic acid.

In this study, several popular metal carbonates with different redox states were used to investigate their performance on bio-succinic acid production from glucose. During fermentation, carbonate serves as the pH buffer and supplies CO2 for the bioconversion of raw materials into succinic acid. The mechanism that governs the performance of these metal carbonates was discussed. *Actinobacillus succinogenes* was chosen as the biocatalyst since it is one of the best succinate producing microbes [10; 11]. Glucose was used as the sole carbon source at a concentration of 30 g/L

2.0 METHODOLOGY

A. succinogenes 130Z was purchased from DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany. The culture was incubated in Brain heart Infusion (BHI) media for 8 hours before it was transferred into fermentation medium. Inoculum (10% v/v) was prepared under aerobic condition at 120 rpm and 37°C. In the fermentation medium of succinic acid, the mineral media consisted of: 15 g/l yeast extract; 3 g KH_2PO_4; 1 g NaCl; 0.2 g $MgCl_2.6H_2O$ and 0.2 g $CaCl_2$; per liter of medium in this study.

Succinic acid fermentation was conducted in batch mode in 110 ml serum vials using 80 ml working volume in sterile condition to study the profile of succinic acid production, byproduct (formate, acetate and ethanol) formation as well as consumption of sugars. Each batch of fermentation was run in triplicate to get the average value. Theoretically, two moles of succinate can be formed from one mole of glucose and two moles of CO_2 [12]. Metal carbonates were supplied at 300 mM, which was in excess on its ratio against 30 g/L of glucose so as to ensure a sufficient supply of CO_2.

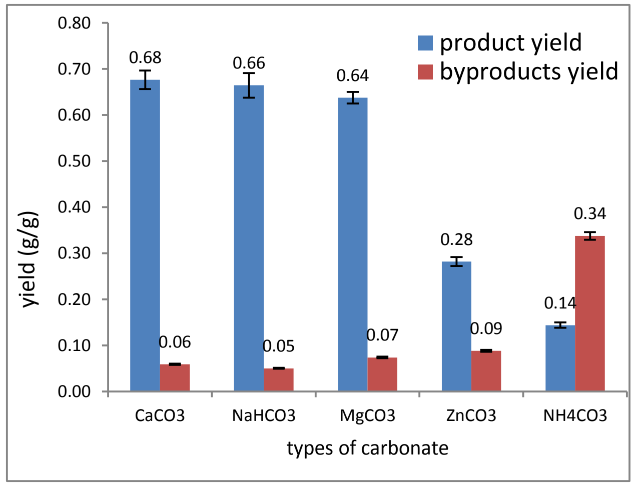


Figure 2 The yield of product and by products

Several metal carbonates including $CaCO_3$, $NaHCO_3$, $MgCO_3$, $ZnCO_3$ and $(NH_4)_2CO_3$ with different reducing power were used to investigate the impact of redox on succinate yield.

In the analysis, glucose was analyzed using Agilent 1200 HPLC system (California, USA) equipped with Rezex RPM (Phenomenex, USA) 300 mm x 7.8 mm column and Refractive Index Detector (RID). Water was isocratically eluted at a flow rate of 0.6 ml/min while the column temperature was set at 60 °C. On the other hand, soluble metabolites such as succinic acid, formic acid and acetic acid were analyzed using Agilent 1100 HPLC system (California, USA) equipped with Rezex ROA column (Phenomenex, USA) and Ultraviolet detector (UVD) at 210 nm. The column was eluted isocratically at a rate of 0.5 ml/min with 0.0025 M H_2SO_4 at 40 °C. Bacteria growth was quantified after the carbonate was dissolved using 0.2 M HCl. The samples were centrifuged and dried in order to quantify its weight in dry basis.

3.0 RESULTS AND DISCUSSION

3.1 Succinic Acid Production

Total sugar consumption was calculated based on the initial and final concentration of glucose analysed using HPLC. Yield was calculated by using the total succinic acid produced divided by total sugar consumed. The total sugar consumption by $CaCO_3$, $NaHCO_3$, $MgCO_3$, $ZnCO_3$ and NH_4CO_3 was as much as 18.9, 27, 29.3, 11.6 and 16.8 g/L respectively as could be inferred from Figure 1. It was shown that $MgCO_3$ had the highest amount of sugar consumed in this study and subsequently generated the highest amount of final succinic acid concentration. This showed that the use of $MgCO_3$ can promote sugar utilization by *A. succinogenes* than others. $MgCO_3$ had the highest succinic acid concentration of 18.7 g/L followed by $NaHCO_3$, $CaCO_3$, $ZnCO_3$ and finally NH_4CO_3 which were 17.9, 12.8, 3.3 12.4 g/L respectively.

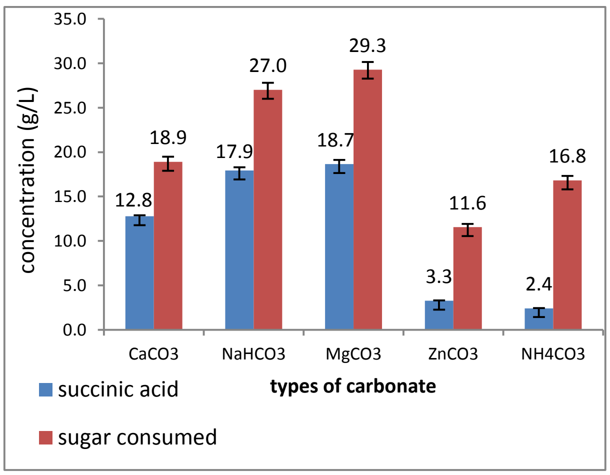


Figure 1 Succinate concentration and total sugar consumption

This final succinic acid concentration trend was largely due to the total sugar consumed. Product and byproducts yield in this regard (Figure 2), gave a better insight to the effect of these metal carbonates on the metabolic fluxes in *A. succinogenes*. The higher yield of succinate implied the higher metabolic flux towards the succinate producing pathway while higher byproducts yield indicated otherwise.

Highest succinic acid yield was shown by $CaCO_3$ with 0.68 g/g followed by $NaHCO_3$ and $MgCO_3$ with 0.66 and 0.64 g/g. On the other hand, $ZnCO_3$ and NH_4CO_3 gave relatively much lower succinic acid yield which were 0.28 and 0.14 g/g respectively. Conversely, the highest byproduct yield was shown by NH_4CO_3 with 0.34 g/g followed by $ZnCO_3$ 0.09g/g. These trends show the metabolic fluxes in *A. succinogenes* which were significantly affected by the redox state of the metal carbonates used.

Eq. (1) to Eq. (5) displayed the reducing power of the metal carbonates relative to H^+ ion. The reducing power of Ca was the highest +2.87 relative to H^+ ion. This is followed by Na, Mg, Zn and lastly NH_4^+. NH_4^+ was in fact had a negative reducing power of -0.27, which implied that NH_4^+ is actually an oxidizing agent to H^+ ion. Higher reducing power of carbonate led to a more reducing fermentation medium. Higher reducing power in turn, triggered the metabolic flux of *A. succinogenes* into the C4 succinate producing pathway [14].

$$Ca^{2+} + 2e^- \rightarrow Ca \quad +2.87 \quad \text{eq (1)}$$
$$Na^{+} + e^- \rightarrow Na \quad +2.71 \quad \text{eq (2)}$$
$$Mg^{2+} + 2e^- \rightarrow Mg \quad +2.37 \quad \text{eq (3)}$$
$$Zn^{2+} + 2e^- \rightarrow Zn \quad +0.76 \quad \text{eq (4)}$$
$$NH^{4+} + e^- \rightarrow NH4 \quad -0.27 \quad \text{eq (5)}$$

3.2 Metabolic Pathways in *A. Succinogenes*

Generally, glucose is consumed by *A. succinogenes* and goes down the glycolysis process until it reaches phosphoenolpyruvate (PEP). PEP serves as the branch between C3-byproducts formation pathway and C4-succinate producing pathway [10]. C3-pathway is an energy generating pathway while C4-pathway is an energy consuming pathway. In the presence of CO_2, PEP undergoes anaplerotic reaction in which CO_2 was incorporated into PEP to form oxaloacetate (OAA). This four carbon OAA will go down the C4-pathway to be further converted into malate, fumarate and finally succinate [13]. Apart from the presence of CO_2, the oxidation reduction potential of the fermentation medium plays an equally important role in promoting the metabolic flux into C4-succinate producing pathway [14]. A more reduced environment will trigger the central metabolic pathway of *A. succinogenes* to be fluxed into C4-pathway, to dispose the excessive reducing power in the form of succinate in order to achieve an intracellular redox balance. This explain the trend of the current results in which higher reductive metal carbonates has a better yield than lower reductive carbonate such as NH_4CO_3.

3.3 Effect of Energy Balance on Stoichiometry Equation

Theoretically, each more of glucose could synthesize two moles of succinate in the excess of CO_2, as shown in eq (6). However, a mole of glucose could produce only two moles of NADH, while four moles of NADH are required to produce two moles of succinic acid. Therefore, two extra moles of NADH are required. By considering oxidation/reduction potentials (ORPs), van Heerden and Nicol proposed [15] an equation stating that one mole of glucose reacts with 6/7 mole of carbon dioxide could produce 12/7 mole of succinic acid and 6/7 mole of water, as shown in eq (7). As a result, maximum yield of succinic acid becomes 1.71 moles per mole of glucose or a yield of 1.12 g/g if it is expressed in mass. This equation ignores the synthesis of biomass and shows the maximum yield of succinic acid possible based on a mole of glucose.

$$C_6H_{12}O_6 + 2\,CO_2 \rightarrow 2\,C_4H_6O_4 + 2\,H_2O \qquad \text{eq (6)}$$

$$C_6H_{12}O_6 + \frac{6}{7}CO_2 \rightarrow \frac{12}{7}C_4H_6O_4 + \frac{6}{7}H_2O \qquad \text{eq (7)}$$

This shows that the availability of reducing power (eg: in the form of NADH) is the bottle neck for higher succinic acid yield. A greater availability of NADH can be achieved by several methods. The use of lower oxidation state carbon sources, the presence of an external reducing agent and lower oxidation/reduction potentials (ORPs) in the medium are among the driving forces toward higher succinic acid yield [16]. This study focused on the effect of metal carbonates with different reducing power on the energy balance that could affect the metabolic fluxes in the succinic acid fermentation.

Biochemical reactions allow the reaction that is extremely slow-occuring in nature to take place. However, they are not able to alter the overall thermodynamics of a chemical reaction [17]. Bacteria will regulate its own energy and redox balance in within the cell. Extracellular redox potential will affect the intracellular reducing equivalents [18]. Microbes (*A. succinogenes*) will be triggered to produce highly reduced metabolites (eg: succinic acid) if the external medium redox potential is lowered. In this way, *A. succinogenes* is able to regain the intracellular redox balance. These are the reasons governing the higher yield of succinate in $CaCO_3$, followed by $NaHCO_3$, $MgCO_3$, $ZnCO_3$ and finally NH_4CO_3 with the lowest reducing power. Therefore, higher succinate yield by $CaCO_3$ (0.68 g/g), which was four times higher than NH_4CO_3 with only 0.14 g/g as could be seen from Figure 1.

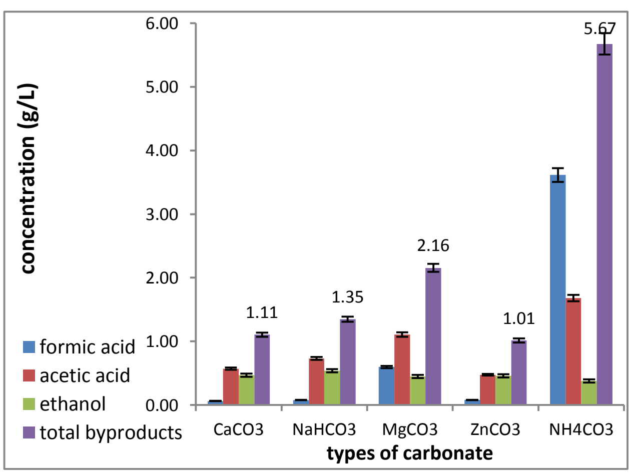


Figure 3 Final byproducts concentration

3.4 Byproducts Production

By-products acetate and succinate have similar properties as succinate since they are all organic acids. This could lead to difficulties during the downstream process. About 50-80 % of the cost of organic acid processing in industries is generally allocated for the product purification [19]. $CaCO_3$ again in this regard, gave an easier downstream process apart from higher succinate yield. This higher final byproducts concentration was due to higher metabolic flux of *A. succinogenes* into C3-byproduct producing pathway. This phenomenon can be observed from the yield of byproducts formate and acetate which was higher in $(NH_4)_2CO_3$.

As shown in Figure 3, $(NH_4)_2CO_3$ marked higher byproducts formation which were 5.67 than $CaCO_3$ which were 1.11 g/g for acetate and formate. NaHCO3, $MgCO_3$ and $ZnCO_3$ on the other hand had shown 1.35, 2.16 and 1.01 g/L of total byproducts

4.0 CONCLUSION

Understanding on the metabolic fluxes allows the manipulation to increase the yield of succinate in fermentation. Biochemical reactions cater naturally slow-occurring but thermodynamically possible reactions. If external reducing power is high, A. succinogenes will be triggered to produce higher reductive metabolites such as succinate to dispose the additional reducing power. This is to allow the bacteria to achieve an intercellular redox balance. Calcium is more reducing than NH4. Therefore, $CaCO_3$ promoted the higher metabolic flux towards succinate rather than byproducts acetate and formate formation. Higher reducing power promoted metabolic flux into highly reducing C4-succinate producing pathway. $MgCO_3$ on the other hand, gave the overall highest amount of succinic acid produced 18.7 g/L due to the higher sugar consumption.

Acknowledgement

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