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# Effect of metal carbonates on succinate production by *Actinobacillus succinogenes* in batch fermentation

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## ABSTRACT

In the present study, the effect of metal carbonates ($CaCO_3$, $MgCO_3$, $MnCO_3$, $Na_2CO_3$ and $K_2CO_3$) on the growth and succinate production by *Actinobacillus succinogenes* 130ZT was examined in 100-mL anaerobic bottles using a glucose-based medium. Initial glucose concentrations and metal carbonate in the form of its chlorides, sulphates, and phosphate were studied. The strain initially produced 7.75 g/L of succinate with a yield of 68% from 15 g/L of glucose within 24 h. However, when process optimization was employed, 10.85 g/L of succinate was produced in the same medium supplemented with 60 mM$MgCO_3$with a succinate yield of 85%. As a result, the titer and yield of succinate were increased by 40% and 31%, respectively. Based on this study, the supplementation of $MgCO_3$ in the cultured medium suggests an effective strategy for improving the succinate production in *A. succinogenes* 130ZT.



## KEYWORDS

*Actinobacillus succinogenes*;
Succinate;
Metal carbonates;
Batch fermentation.

## INTRODUCTION

Succinate is a four-carbons dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle[1]. It serves as a precursor to various commodity chemicals used in industry including adipic acid, 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, γ-butyrolactone, poly-butyrate succinate, polyamides, and various green solvents[2,3]. Traditionally, succinate has been acquired from petrochemicals in which the process is costly and causes pollution. The production of succinate from a naturally-derived biomass would alleviate the independence on oil supply in the future. In contrast, microbial conversion of biomass into succinate has been increasingly attractive as an eco-friendly and energy-saving process[4]. The capnophilic bacterium, *Actinobacillus succinogenes*, is considered as a succinate producer because of its ability to pro-

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duce a large amount of succinate, to use a wide range of carbon sources, and to tolerate high concentrations of organic acids[5].

It has been reported that the level of $CO_2$ in the medium exerts a significant effect on the succinate production in other succinate producing bacteria including *Mannheimia succiniciproducens*[6], *Anaerobiospirillum succiniciproducens*[7], *Bacteroides fragilis*[8], and *Enterococcus flavescens*[9]. A few literatures have suggested that metal carbonate, which represents the source of $CO_2$, is the most factors influencing both cell growth and the succinate production in many succinate-producing bacteria[8-10]. However, the strategy of enhancing the supply of metal carbonates for the succinate production of *A. succinogenes* 130ZT has not been yet investigated. Thus, the objective of this study was to determine the effect of different metal carbonates and their concentrations on the cell growth and the succinate production in terms of titer, yield, and productivities by *A. succinogenes* 130ZT.

## MATERIAL AND METHODS

### Chemicals and gas

All chemicals used for media preparation were an analytical grade and were purchased from the local commercial sources. High purity of carbon dioxide gas was purchased from Thailand industrial gas Co., Ltd. Thailand.

### Organism, media and seed culture preparation

*A. succinogenes* 130ZT (DSM 22257) was used throughout this study. The fermentation medium was composed of (per liter) 5 g yeast extract, 10 g $KHCO_3$, 8.5 g $NaH_2PO_4$, 15.5 g $K_2HPO_4$, 0.05 g $MgSO_4\,7H_2O$, and 1 g NaCl. The pH of the medium was adjusted to 7.5 by NaOH before sterilization. The strain was precultured in 100 mL sealed-anaerobic bottles containing 50 mL of the medium. Five grams per liter of glucose was added separately to the medium after autoclaving. For the seed culture, 50 µL of the stock culture was inoculated in the sterile medium. The medium was purged by an aseptic $CO_2$ gas for 2 min to generate an anaerobic environment. The seed culture was incubated at 37 ºC, 100 rpm for 16-18 h. The fermentation culture was inoculated with 6% (w/v) seed culture and incubated at 37 ºC, 100 rpm for 24 h.

### Batch fermentation

Batch fermentation was carried out in sealed 100-mL anaerobic bottle containing 50 mL culture medium. The different concentrations of glucose were first investigated in the range of 1 to 50 g/L. Subsequently, the different metal carbonates ($CaCO_3$, $MgCO_3$, $MnCO_3$, $Na_2CO_3$ and $K_2CO_3$ at the concentration of 20 mM) were added in the optimized medium to assess their effect on the production of succinate. Further, the effect of different salts of the metal ion of the best metal carbonate was studied in the form of its chlorides, sulphates, and phosphate (20 mM). Finally, the suitable carbonate concentration was determined the effect of its concentrations on the succinate production. All the experiments were performed in triplicates and results are presented along with standard deviation.

### Analytical methods

One milliliter of the culture was centrifuged at 13,500 rpm for 10 min. The supernatant was further analyzed for the fermentative products. Biomass concentration was determined by measuring the optical density at 550 nm using a spectrophotometer (Spekol-1500, Anatytik Jena, Thailand). The optical densities were then converted to dry cell weight (OD 1.0 = 0.333 mg of cell dry weight/L), and defined as biomass concentration. succinate concentration was analyzed by high performance liquid chromatography (Agilent technology, Japan) equipped with an ion exclusion column (BIO RAD, Aminex, HPX-87H, USA) with a column temperature of 45 ºC using 4 mM $H_2SO_4$ as a mobile phase with a flow rate of 0.4 mL/min).

The yield of succinate was defined as the amount of succinate in gram formed per one gram ofglucose consumed. Succinate productivity was calculated as the maximum succinate concentration produced within a total time incubated (g/L/h).

## RESULTS AND DISCUSSION

### Effect of glucose concentrations on the succinate production

The cell growth and succinate production were examined in the medium containing different initial glucose concentrations. Succinate and biomass productions

were increased when initial concentrations of glucose were gradually supplemented. However, a decrease in the succinate and biomass production was observed when the initial glucose concentration was greater than 15 g/L (Figure 1). The growth of this microorganism started as early as 12 h, but reached at maximum within 24 h, and was constantly remained thereafter (Figure 2). Succinate production also started as early as 12 h and reached the maximal level at 7.75 g/L after 24 h with yield and productivity of 68% and 0.32 g/L/h, respectively. The maximum succinate concentration of 7.75 g/L was obtained when the initial concentration of glucose was 15 g/L. Therefore, it was likely that the cell growth and succinate production were inhibited by high glucose concentration presented in the medium. It has also been demonstrated that the decrease in the succinate production was due to high osmotic pressure causing from high glucose concentration. This also affected the enzymatic activity of enzymes involving in the succinate production[8,10]. Therefore, it suggested that the optimized concentration of glucose for the succinate production by this *A. succinogenes* 130ZT was 15 g/L. This glucose concentration was used further for subsequent study.

## Effect of metal carbonates on the succinate production

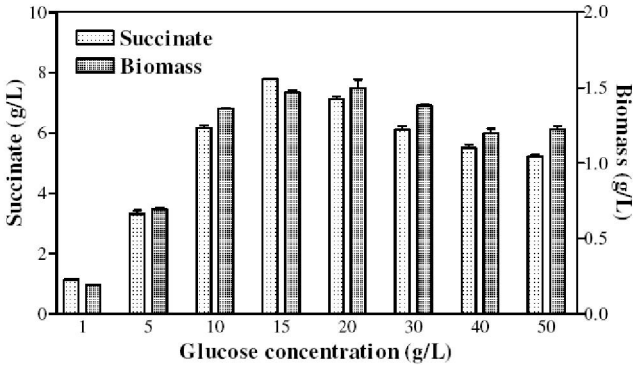


**Figure 1 : Effect of different concentration of glucose on succinate production from *A. succinogenes* 130ZT in 100-mL anaerobic bottle after 24 h of incubation**

Metal ions are known to play an important role in maintaining cellular metabolism and enzymatic activities. Therefore, the effect of metal carbonate on the succinate production by *A. succinogenes* 130ZT was also determined. A significant increase in the production of succinate (8.45 g/L) was achieved when the medium was supplemented with $MgCO_3$. It could be the results that the availability of magnesium ions plays an important role in maintaining cellular metabolism in which the ion is a cofactor for most of the enzymes involved in the succinate production[11]. Similarly, the maximum level of succinate (5.32 g/L) produced by *En. flavescens* was obtained when $MgCO_3$ was added in the culture medium[9]. This is in agreement with *E. coli* that the rate of PEP carboxylase activity for substituted cation $Mg^{2+}$ ions was higher than that of other metal ions like $Mn^{2+}$, $Co^{2+}$, or $Zn^{2+}$[12]. Also, Podkovyrov and Zeikus (1993) reported that an *in vitro* activity of PEP carboxykinase purified from *An. succiniciproducens* was significantly enhanced by $Mg^{2+}$ ions[13,14].

## Effect of metal ions on succinate production

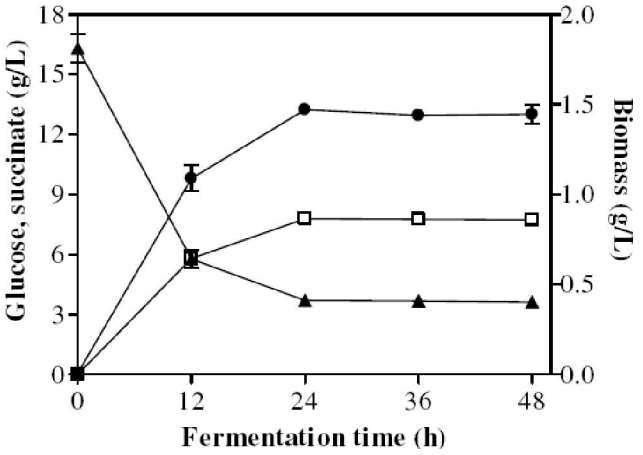


**Figure 2 : Fermentation kinetic of *A. succinogenes* 130ZT on glucose medium, Glucose used was 15 g/L. Symbol represent; (▲) glucose (g/L), (•) biomass (g/L), (□) succinate (g/L)**

It was hypothesized that the metal ions or carbonate ions affect the succinate production. Then the effects of different magnesium salts on the succinate production by *A. succinogenes* 130ZT were further investigated. As shown in Figure 4, $MgCO_3$ was the most effective for the succinate production resulting in 8.45 g/L of succinate compared to $MgCl_2$, $MgSO_4$, and $MgPO_4$ in which the succinate concentrations of 7.85, 7.75, and 7.58 g/L, respectively, were observed. It is most likely that the supply of $CO_2$ during the fermentation process is significant for the succinate production. Van der Werf *et al*. (1997) and Samuelov *et al*. (1991) explained that the production of succinate requires $CO_2$ fixation and the $CO_2$ concentration regulates the level

of key enzymes of the PEP carboxykinase pathway in *An. succiniciproducens*[15,16]. They further confirmed that high levels of $CO_2$ stimulated PEP carboxykinase levels, whereas the levels of alcohol dehydrogenase and lactate dehydrogenases were significantly decreased. Zeikus *et al*. (1999) also demonstrated that $CO_2$ functions as an electron acceptor and alters the flux of PEP, which metabolizes to pyruvate and lactate/ethanol at low $CO_2$ levels but makes succinate at high $CO_2$ concentration[17].

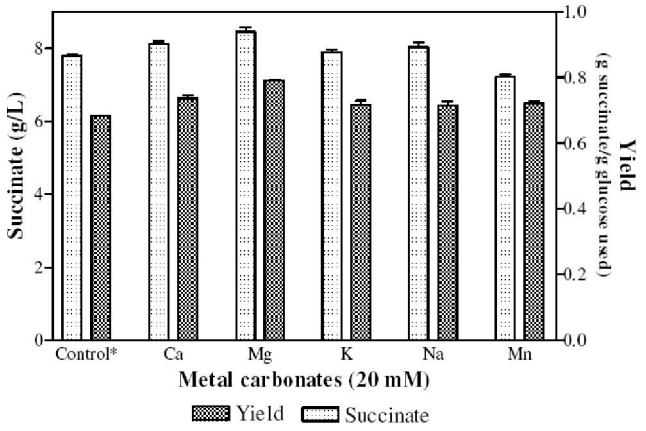


**Figure 3 : Effect of different metal carbonates on succinate production from *A. succinogenes*130ZT in 100-mL anaerobic bottle after 24 h of incubation. *Control, the medium without addition of extra metal carbonates**

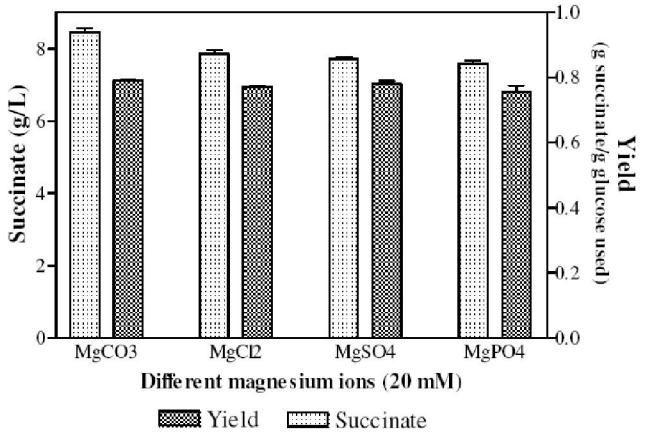


**Figure 4 : Effect of different magnesium ions on succinate production from *A. succinogenes*130ZT in 100-mL anaerobic bottle after 24 h of incubation**

## Effect of magnesium carbonate concentrations on succinate production

With a view to maximize the succinate concentration, the effect of $MgCO_3$ concentration on the succinate production was subsequently investigated. It could be observed that a significant increase in the succinate production (10.85 g/L) by the strain was achieved within 24 h when the medium was supplemented with 60 mM of $MgCO_3$. However, further increase in $MgCO_3$ concentration did not improve the production of succinate. This might be explained that a higher released $CO_2$ caused an acid formation during fermentation. Increase in $MgCO_3$ concentration indicated increased $CO_2$ source. Nevertheless, Wang *et al*. (2009) observed that glucose consumption and succinate production in *E. coli* were suppressed to some extent in the medium supplemented with $MgCO_3$ at the concentration greater than 20 g/L[18]. Also, Kwon *et al*. (2007) found that *E. coli* confers the upper limit for bicarbonate at the level of 10 g/L for the succinate production[14]. So it was important to note that the carbonate concentration in the fermentation broth should be optimized. Based on these results, the supplementation of an optimum concentration of $MgCO_3$ at 60 mM resulted in increasing of titer and succinate yield by 40% and 30.77%, respectively. These results indicated that the supplementation of $MgCO_3$ is critical for enhancing the succinate production in *A. succinogenes*130ZT.

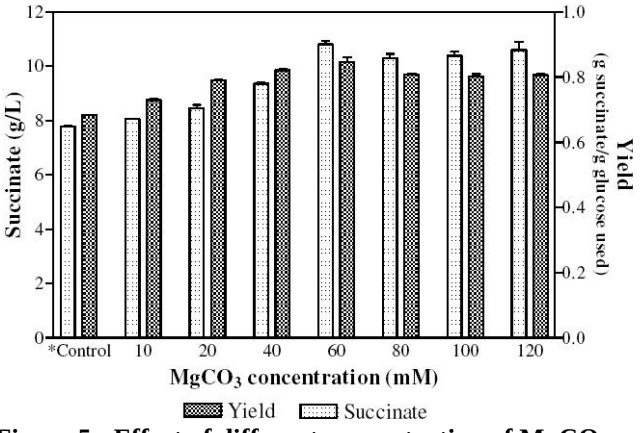


**Figure 5 : Effect of different concentration of $MgCO_3$ on succinate production from *A. succinogenes*130ZT in 100-mL anaerobic bottle after 24 h of incubation. *Control, the medium without addition of extra metal carbonates**

## CONCLUSION

This study demonstrated that the succinate production could be enhanced by supplementing of $MgCO_3$ at 60 mM in the medium containing 15 g/L glucose. The succinate concentration at 10.85 g/L with a yield of 85% was obtained. As a result, the titer and yield of succi-

nate were increased by 40% and 30.77%, respectively. The results obtained from this study may help in the design of a new strategy for an efficient succinate production by *A. succinogenes* 130ZT at the large scale.

## ACKNOWLEDGEMENTS

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