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Potential industrial application of *Actinobacillus succinogenes* NJ113 for pyruvic acid production by microaerobic fermentation

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Abstract−*Actinobacillus succinogenes* NJ113 is capable of microaerobic fermentation, which offers the possibility of a novel type of pyruvic acid production. A dissolved oxygen environment with stirring at 300 rpm was a key factor in the fermentative production of a maximum concentration of pyruvic acid. Potassium carbonate (K_2CO_3) was found to have a role in promoting pyruvic acid production, influencing the concentration of pyruvic acid and production of the by-product succinic acid. The final titer of pyruvic acid production was 36.8±0.1 g L^{-1} with an overall yield of 0.639±0.056 g g^{-1} glucose and 3.12±0.03 mmol g^{-1} dry cell weight h^{-1}.

Significance and impact of the study: This study is the first to illustrate the advantage of using Actinobacillus succinogenes NJ113 with no genetic modification under microaerobic conditions for the production of pyruvic acid.

Keywords: *Actinobacillus succinogenes*, Dissolved Oxygen Environment, Microaerobic Fermentation, Potassium Carbonate, Pyruvic Acid

INTRODUCTION

The bioproduction of organic acids is no longer a theoretical topic; rather, it is in use on a commercial scale. The beginnings of industrialization of bioproduction can be found in the biosynthesis of citric acid in 1919 by Charles Pfizer [1]. This suggested biosynthesis by microorganisms could be a low-cost, efficient and environment friendly way to produce organic acids [2]. Owing to the development of biosynthesis, bioproducts are used in many aspects of human society, including textiles and the food industry, pharmaceuticals industry, metallurgical industry, petroleum chemistry industry etc.

The commercial demand for pyruvic acid has been increasing because of its use as an effective precursor in the synthesis of various chemicals and polymers as well as an additive or ingredient in food, cosmetics and pharmaceuticals [3,4]. Further, it occupies an important place in weight loss diets and exercise endurance as well as anti-acne and anti-ageing skin treatment, and it serves as an antioxidant and acts positively on graft tolerance [3]. The chemical production of pyruvate by tartaric acid is not very cost efficient [5], and therefore the possibility of biological production of pyruvate has attracted a great deal of interest and has been implemented successfully with metabolically engineered *Escherichia coli* strains [6], multi-auxotrophic yeasts [4] and engineered *Corynebacterium glutamicum* [7].

Since *Actinobacillus succinogenes*, a facultatively anaerobic microorganism, was found, a wide variety of interesting metabolic features have been identified [8]. *A. succinogenes* was isolated originally from bovine rumen and is considered one of the most promising organisms for the industrial-scale production of succinic acid [9]. *A. succinogenes* can produce a high titer and a high yield of lactic acid at the end of dual-phase fermentation [10]. Lactate dehydrogenase activity in the dual-phase process was nearly 18-fold greater compared to the mono-phase process and, as a result, lactic acid production increased 32-fold, up to a final titer of 135.6 g L^{-1} [10].

A. succinogenes has industrial potential as well as unique metabolic traits that could contribute to a broader understanding of bacterial metabolism and its diversity [8]. To our knowledge, however, targeted production of pyruvate with *A. succinogenes* has not been reported, and there has been no effort to produce pyruvate with this organism. In the work reported here, we used optimized conditions to obtain pyruvate production by *A. succinogenes* NJ113 with no genetic modification. The influence of a dissolved oxygen environment and pH buffers on the biosynthesis of pyruvate by *A. succinogenes* was studied with the aim of establishing optimum fermentation conditions.

MATERIALS AND METHODS

1. Chemicals and Gas

Yeast Extract was from Oxoid Ltd (Cambridge, UK). All other chemicals were of reagent grade and purchased from Sinochem (Shanghai, China) or Fluka Chemical (Buchs, Switzerland). Carbon dioxide $(CO_2)_{gas}$ and nitrogen $(N_2)_{gas}$ were purchased from Nanjing Special Gases Factory (Nanjing, China).

2. Microorganism and Media

A. succinogenes NJ113 (China General Microbiological Culture Collection Centre, CGMCC no. 1716) was used in all experiments.

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The medium for the inoculation cultures was composed of (in g L^{-1}) glucose (10), yeast extract (5), $NaH_2PO_4 \cdot 2H_2O$ (9.6), $K_2HPO_4 \cdot 3H_2O$ (15.5), $NaHCO_3$ (10), NaCl (1) and corn steep liquor (2.5). The fermentation medium contained (in g L^{-1}) glucose (50), yeast extract (10), KH_2PO_4 (3), $MgCl_2 \cdot 6H_2O$ (0.3), $CaCl_2$ (0.3), NaCl (1), $NaHPO_4 \cdot 12H_2O$ (0.31) and $NaH_2PO_4 \cdot 2H_2O$ (1.6).

3. Growth Conditions and Fermentation in Stirred Bioreactors

The inoculation medium was mixed with 1 mL of a glycerol stock culture stored at −70 °C and then incubated under anaerobic conditions at 37 °C with stirring at 200 rpm.

Fed-batch fermentation was at 37 °C in a 7.5 L stirred bioreactor (New Brunswick Scientific Co., Inc., NJ 08817, USA) with an initial broth volume of 3 L of fermentation medium with the pH adjusted to 6.8. The inoculation defined the quantity in controlled in 10%. When the glucose concentration was <5 g L^{-1}, glucose (600 g L^{-1}) was fed into the fermentation medium until the final concentration was 30 g L^{-1}.

4. Aerobic, Anaerobic and Microaerobic Fermentation

Under aerobic fermentation conditions, dissolved oxygen levels were 100% of air saturation at the time of inoculation and were allowed to fall to 40% of air saturation during cell growth with continuous air sparging (1 vessel volume min^{-1}). The pH of the broth was controlled with 20% (w/v) Na_2CO_3. Under anaerobic fermentation conditions, nitrogen gas was bubbled through the medium for 60 min to remove oxygen before inoculation. The pH of the broth was controlled with 20% Na_2CO_3. Under microaerobic fermentation conditions, the dissolved oxygen level was 100% of air saturation at the time of inoculation with continuous air sparging (1 vessel volume min^{-1}) and dropped below 5% after fermentation for 5 h.

5. Analytical Methods

Dry cell weight (DCW) was computed from a curve relating the optical density at 660 nm (OD_{660}) to DCW. An OD_{660} value of 1.0 represented 520 mg of DCW L^{-1} [11]. Glucose was measured using an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, China). The concentrations of organic acids were measured by high-performance liquid chromatography using an Agilent 1290 instrument (Agilent, Co. Ltd., CA, USA) equipped with an Agilent variable wave detector and an HPX-87H column (300 mm×7.8 mm; BIO-RAD). The column temperature was 55 °C, the mobile phase was 0.008 M sulfuric acid at a flow rate of 0.6 mL min^{-1}, and the injection volume was 20 μL. The intracellular concentrations of NADH and NAD^+ were measured using a cycling method [12].

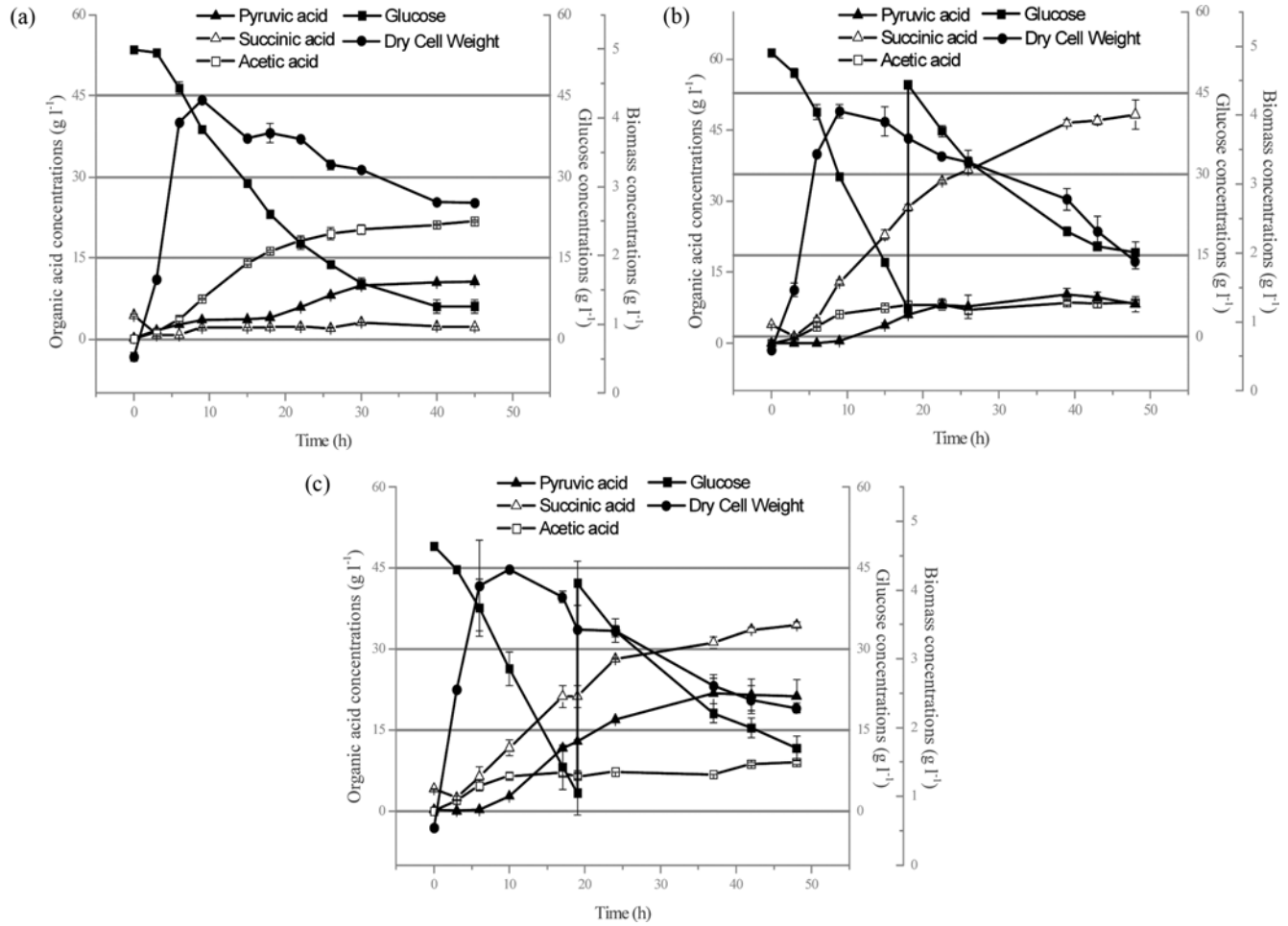

Fig. 1. Organic acids produced by *A. succinogenes* NJ113 under different cultivation modes: (a) Aerobic fermentation; (b) anaerobic fermentation; (c) microaerobic fermentation (All data are corrected for the influence of pH buffers on volume.).

RESULTS AND DISCUSSION

1. Organic Acids Produced under Different Cultivation Modes

Facultatively anaerobic microorganisms are able to grow under aerobic and anaerobic conditions by changing their cell physiology and metabolic pathways to adapt to the environment [13,14]. The range of organic acids produced is controlled by sensing and regulatory systems that sense oxygen levels and transmit a signal to modify gene expression accordingly [15]. Therefore, different cultivation modes will influence the range of organic acids produced by fermentation. The effects of different cultivation modes with *A. succinogenes* under anaerobic, aerobic and microaerobic fermentation were investigated in a 7.5 L fermentor using Na_2CO_3 as a pH modifier.

As shown in Fig. 1, during aerobic fermentation with dissolved oxygen at 40% (Fig. 1(a)), 21.8 g L^{-1} of acetic acid was the main metabolite with pyruvic acid (10.6 g L^{-1}) as the by-product. Succinic acid was not detected and the rate of glucose consumption was slow. Further, the cell growth rate was higher and more stable under aerobic conditions compared to anaerobic (Fig. 1(b)) and microaerobic conditions (Fig. 1(c)). Cell growth and glucose consumption were similar during microaerobic fermentation and anaerobic fermentation, but pyruvic acid production during microaerobic fermentation was increased to 21.4 g L^{-1}, which was much higher compared to anaerobic fermentation (8.3 g L^{-1}). As a result, the concentration of pyruvic acid under microaerobic conditions increased to 158% and 102% compared to anaerobic and aerobic fermentation, respectively.

All these findings throw light on the potential role of *A. succinogenes* in the bioproduction of pyruvic acid. The results given above led us to assume there is a correlation between cultivation modes (i.e., the dissolved oxygen environment) and pyruvic acid production. At present, we have detailed knowledge of the metabolic pathways and fluxes of *A. succinogenes* under anaerobic conditions [16]. The concentration of CO_2 affects the flux distribution into C_4 and C_3 pathways and this can occur at multiple nodes (i.e., phosphoenolpyruvate, oxaloacetate, malate and, possibly, pyruvate) [16]. Therefore, variation of the dissolved oxygen environment and pH buffers was evaluated.

2. Pyruvic Acid Bioproduction in Different Dissolved Oxygen Environments

As mentioned above, a dissolved oxygen environment was one of the critical factors for the accumulation of the metabolic intermediate pyruvic acid via aerobic, anaerobic and microaerobic fer-

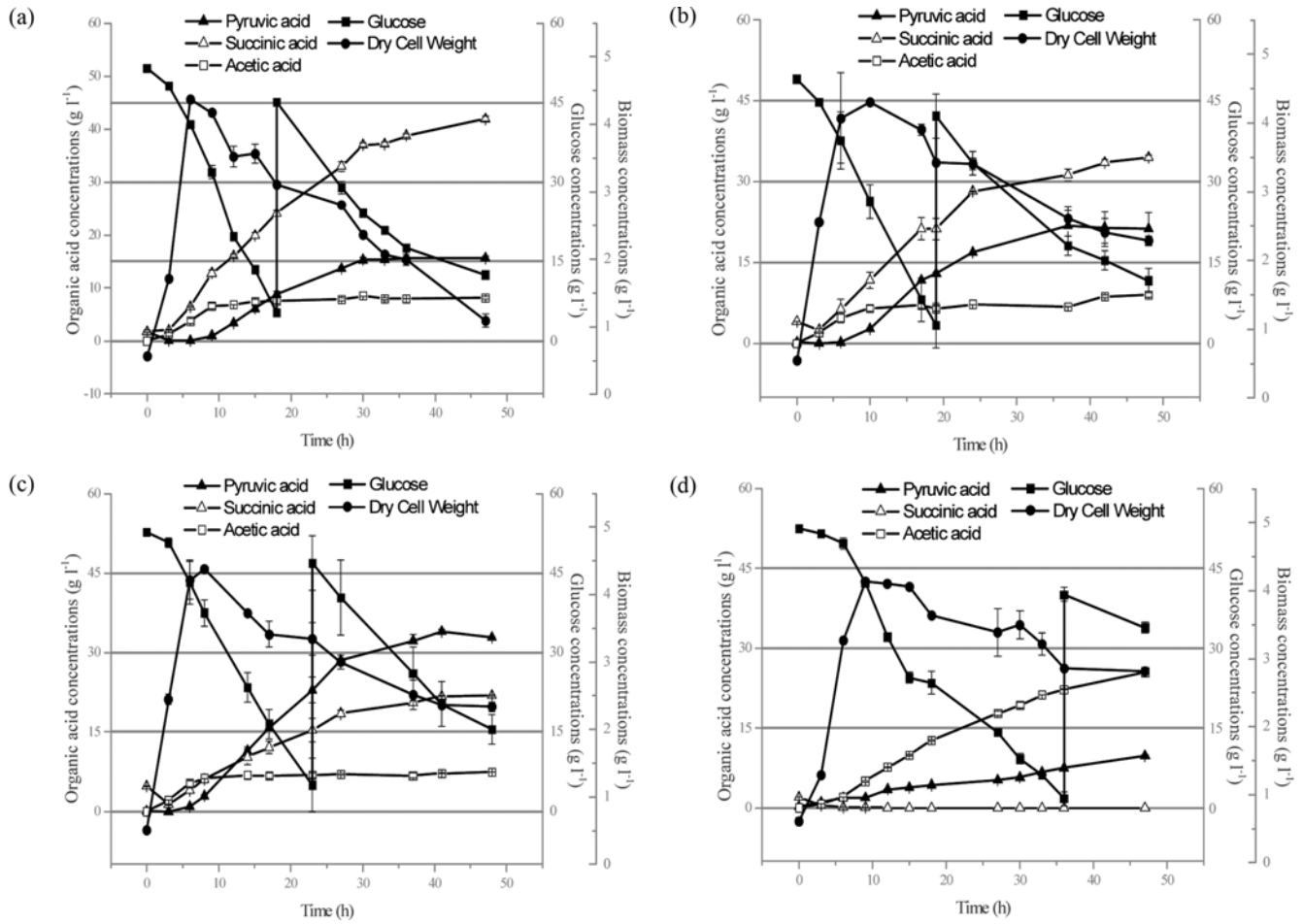

Fig. 2. Organic acids produced by *A. succinogenes* NJ113 under different dissolved oxygen environments with stirring at: (a) 100 rpm; (b) 200 rpm; (c) 300 rpm and (d) 400 rpm.

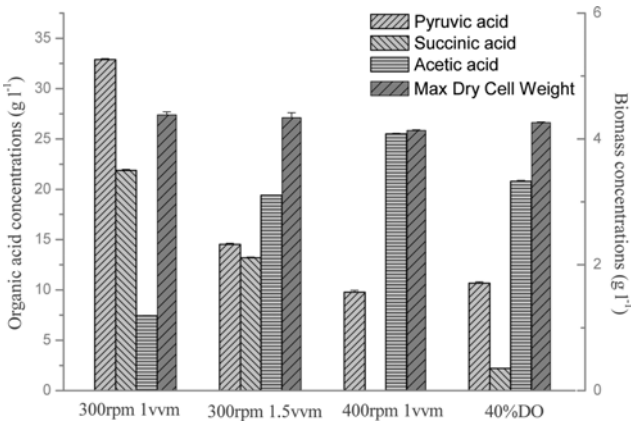

Fig. 3. Organic acids produced by *A. succinogenes* NJ113 under different dissolved oxygen environments with stirring at: 300 rpm 1 vvm (vessel volume min^{-1}); 300 rpm 1.5 vvm; 400 rpm 1 vvm; and 40% DO.

mentation. The concentration of dissolved oxygen available for microaerobic fermentation was <5%, so it was difficult to make an accurate control strategy. Accordingly, we investigated the design of control strategies with differing stirring speeds (Fig. 2).

As shown in Fig. 2, cell growth was increasingly stable, but the concentration of succinate decreased when stirring speed was increased from 100 to 400 rpm. There was no significant change of the concentration of acetic acid with stirring speed increasing from 100 to 300 rpm; however, acetic acid was the main metabolite (25.5 g L^{-1}) when the stirring speed was increased to 400 rpm. The concentration of metabolically produced pyruvic acid increased and a maximum concentration of 32.9 g L^{-1} was obtained at a stirring speed of 300 rpm. When the stirring speed was increased from 300 to 400 rpm, the concentration of pyruvic acid decreased to 9.79 g L^{-1}. The concentration, the yield (0.415 g/g) and the productivity (3.02 mmol g^{-1} DCW h^{-1}) of pyruvic acid were greatest when the stirring speed was 300 rpm.

As shown in Fig. 3, the concentration of acetic acid was increased, whereas the concentrations of succinate and pyruvic acid decreased under aerobic conditions compared to microaerobic conditions (stirring speed 300 rpm). Under microaerobic conditions, the same trend was observed when air sparging and stirring speed increased. Thus, a dissolved oxygen environment regulated by air sparging or stirring speed was one of the important factors for the accumulation of pyruvic acid.

The low production capacity of succinate acid and acetic acid led to the accumulation of pyruvic acid under microaerobic conditions. As shown in Fig. 4, phosphoenolpyruvate, oxaloacetate and malate can give rise to the enrichment of pyruvic acid. Under microaerobic conditions, the presence of molecular oxygen decreases the production of succinate, which increases flux distribution into pyruvic acid [17]. This conclusion fit well with the concentrations of NADH and NAD^+. The radio of NADH/NAD^+ was higher under anaerobic conditions (0.95±0.08) than microaerobic conditions (0.08±0.02) after fermentation for 23 h because succinic acid accumulation was inhibited. The same features might exist under microaerobic conditions, where the low levels of activity of pyruvate

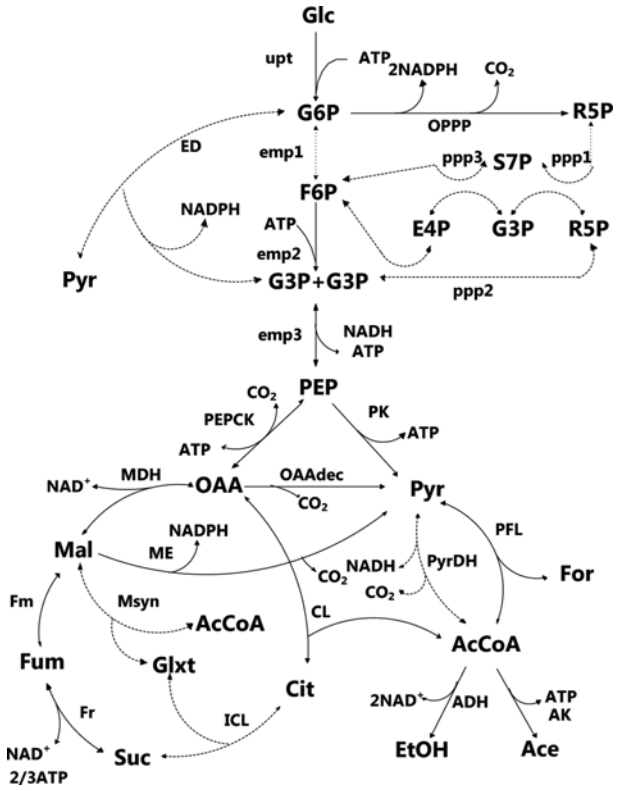

Fig. 4. *A. succinogenes* metabolic pathways [16]. Unbroken lines, pathways or reactions for which enzyme activity was detected in vitro; dotted lines, pathways or reactions where no activity or uncertain activity was detected in vitro; unidirectional arrows, fluxes considered to be unidirectional (all other fluxes are considered to be reversible). The C_4 pathway is defined as: PEP→OAA→malate→fumarate→succinate. The C_3 pathway is defined as: PEP→pyruvate→acetyl CoA→acetate+ethanol. Metabolites: AcCoA, acetyl-coenzymeA; Ace, acetate; Cit, citrate; EtOH, ethanol; E4P, erythrose-4-phosphate; For, formate; Fum, fumarate; F6P, fructose-6-phosphate; Glc, glucose; Glxt, glyoxylate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; R5P, pentose-phosphates, Suc, succinate; S7P, sedoheptulose-7-phosphate. Pathways and reactions: ADH, alcohol dehydrogenase; AK, acetate kinase; CL, citrate lyase; ED, Entner-Doudoroff pathway; emp1, 2, and 3, Embden-Meyerhoff-Parnas (EMP) or glycolytic reactions; Fm, fumarase; FR, fumarate reductase; ICL, isocitrate lyase and aconitase; MDH, malate dehydrogenase; ME, malic enzyme; Msyn, malate synthase; OAAdec, oxaloacetate decarboxylase; OPPP, oxidative pentose phosphate pathway; PEPCK, PEP carboxykinase; PFL, pyruvate formate-lyase; PK, pyruvate kinase and PEP:glucose phosphotransferase system (PTS); ppp1 and 2, transketolase; ppp3, transaldolase; PyrDH, pyruvate dehydrogenase or PFL coupled with formate dehydrogenase; upt, glucose phosphorylation by hexokinase and PTS.

dehydrogenase and formate dehydrogenase detected under anaerobic conditions lead to the accumulation of pyruvic acid [16]. With increased dissolved oxygen, pyruvic acid might be decomposed to acetic acid [18].

3. Pyruvic Acid Production with Different pH Buffers

Different pH buffers, including carbonate and metal ions, have

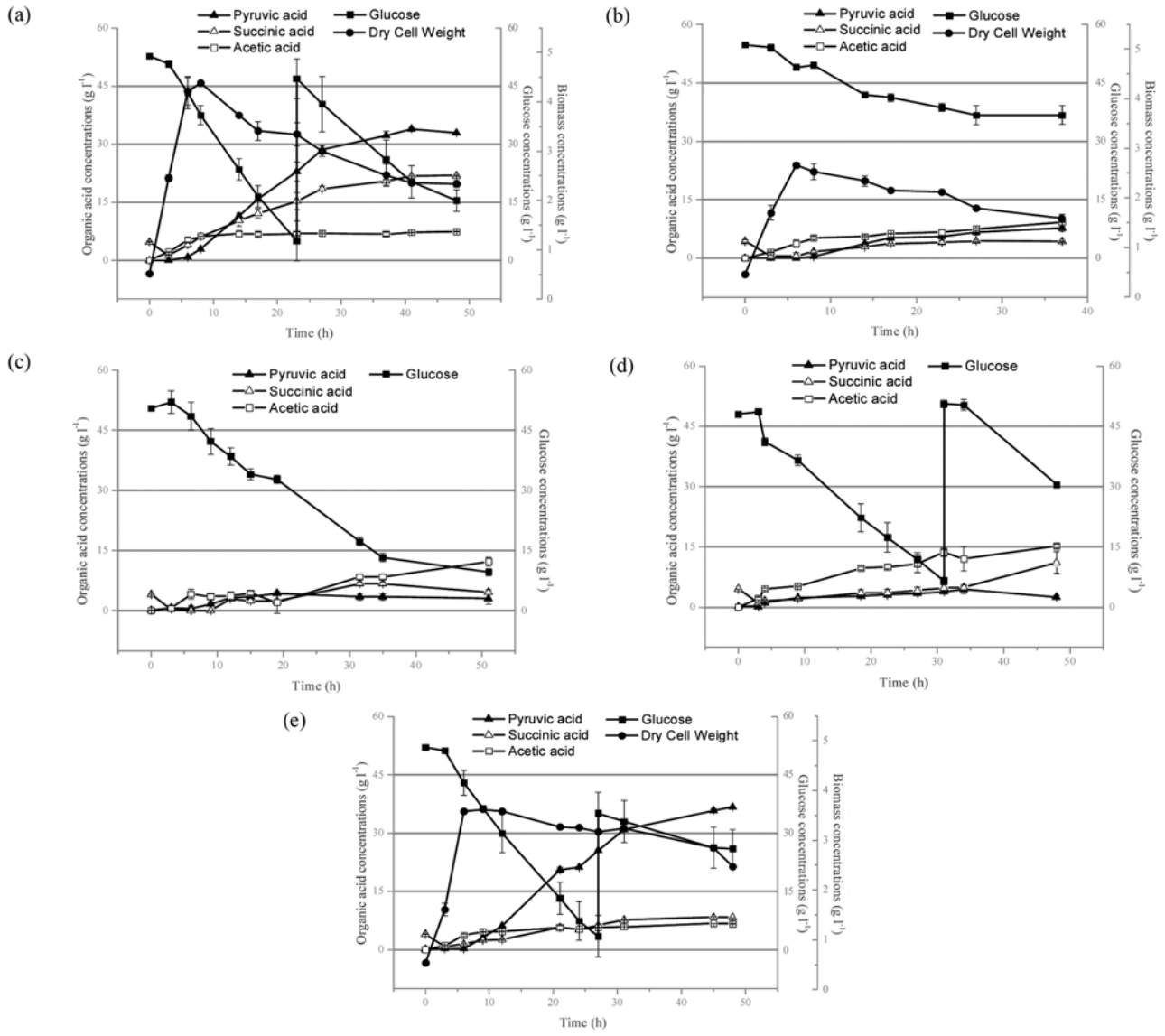

Fig. 5. Organic acids produced by *A. succinogenes* NJ113 with different pH buffers: (a) Na_2CO_3; (b) NaOH; (c) $MgCO_3$; (d) Na_2CO_3+$Mg(OH)_2$; and (e) K_2CO_3.

significant influence on succinic acid production under anaerobic conditions. Under microaerobic conditions, however, the effect of pH buffers on pyruvic acid production was not known and was investigated in this study (Fig. 5).

As shown in Fig. 5, compared with Na_2CO_3 (Fig. 5(a)), low DCW, low glucose consumption and low organic acid production were obtained with NaOH (Fig. 5(b)) as the pH buffer. Therefore, the carbonate ion was an indispensable part of growth and fermentation to produce organic acids. As shown in Fig. 6, cell growth as well as the concentration of succinate and pyruvic acid were increased as the concentration of carbonate increased.

In addition, magnesium ions had no role for organic acid production under microaerobic conditions according to fermentation with $MgCO_3$ (Fig. 5(c)) and Na_2CO_3 combined with $Mg(OH)_2$ (Fig. 5(d)) as pH buffers. However, the K_2CO_3 pH buffer (Fig. 5(e)) was fermented efficiently by *A. succinogenes* NJ113 to produce pyruvic acid with a yield of 63.9%, and the pyruvic acid concentration, yield

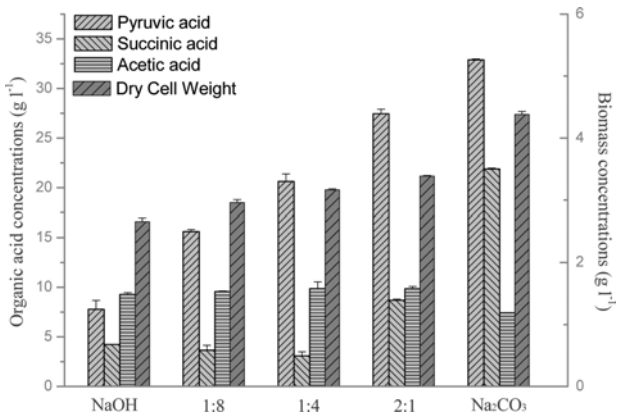

Fig. 6. Organic acids produced by *A. succinogenes* NJ113 using different concentrations of sodium carbonate with NaOH as pH buffer: NaOH; 1 : 8 (NaOH : Na_2CO_3); 1 : 4 (NaOH : Na_2CO_3); 2 : 1 (NaOH : Na_2CO_3); and Na_2CO_3.

Table 1. Different strains produced pyruvic acid. Genetic modification-type microbial strain: *S. cerevisiae* TAM; *E. coli* YYC202 ldhA::Kan. Wild-type microbial strain: *Acinetobacter* sp. 80-M; *Enterococcus casseliflavus A-12*; *A. succinogenes* NJ113 (this study)

Strain	Characteristic	Pyruvate ($g\ L^{-1}$)	Yield glucose ($g\ g^{-1}$ DCW)	Productivity (mmol g^{-1} DCW·h^{-1})
S. cerevisiae TAM	Glucose-tolerant and acetate-independent mutant of pyruvate decarboxylase-negative	135	0.54	6
E. coli YYC202 ldhA::Kan	Mutant of *E. coli* YYC202 in which lactate dehydrogenase (ldhA), responsible for the conversion of pyruvate into lactate, is inactivated	79	0.87	5.72
Acinetobacter sp. 80-M	B1-	11.6	0.58 (1,2-Propanediol)	/
Enterococcus casseliflavus A-12	NO	16	0.32	/
A. succinogenes NJ113	NO	36.8	0.637	3.12

and productivity were increased to 11.9%, 54.0% and 3.31%, respectively, and the by-product succinic acid was decreased by 164.2% compared to Na_2CO_3 as the pH buffer.

Fermentation with K_2CO_3 as the pH buffer compared to other pyruvic acid production strains is given in Table 1. Among the published processes, the Maris group obtained the highest concentration (135.0 g L^{-1}) of pyruvic acid by directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae* TAM with a specific pyruvic acid production rate of 6 mmol g^{-1} DCW h^{-1} and an overall yield of 0.54% [19]. In prokaryotes, the pyruvate production strain *Escherichia coli* YYC202 ldhA::Kan is blocked completely in its ability to convert pyruvate into acetyl-CoA or acetate, resulting in acetate auxotrophy during growth in glucose minimal medium. A final concentration of pyruvic acid of 79 g L^{-1} was obtained under optimum conditions [20]. By contrast, even the concentration of pyruvic acid produced by *A. succinogenes* NJ113 and the productivity were not high but the yield had increased. In addition, *A. succinogenes* NJ113 has an advantage in terms of the concentration of pyruvic acid and the yield compared to other wild type strains producing pyruvic acid, including *Acinetobacter* sp. 80-M and *Enterococcus casseliflavus* A-12 [21,22]. Therefore, *A. succinogenes* NJ113 with no genetic modification has excellent potential for the bioproduction of pyruvic acid.

Different cultivation modes are the key to the distribution of organic acids and the fermentative production of pyruvic acid. Therefore, an appropriate dissolved oxygen environment is important for the economic production of pyruvic acid. This study describes the influence of cultivation modes, dissolved oxygen environment and pH buffers on the biosynthesis of pyruvate by *A. succinogenes* NJ113. The results indicated *A. succinogenes* NJ113 is potentially an excellent strain for the bioproduction of pyruvic acid.

ACKNOWLEDGEMENTS

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REFERENCES

1. G. C. Paul and C. R. Thomas, *Adv. Biochem. Eng./Biotechnol.*, **60**, 1 (1998).
2. J. B. McKinlay, C. Vieille and J. G. Zeikus, *Appl. Microbiol. Biotechnol.*, **76**, 727 (2007).
3. Y. Li, J. Chen and S. Y. Lun, *Appl. Microbiol. Biotechnol.*, **57**, 451 (2001).
4. Q. Wang, P. He, D. Lu, A. Shen and N. Jiang, *Lett. Appl. Microbiol.*, **35**, 338 (2002).
5. P. Xu, J. H. Qiu, C. Gao and C. Q. Ma, *J. Bioscience Bioengineering*, **105**, 169 (2008).
6. V. F. Wendisch, M. Bott and B. J. Eikmanns, *Current Opinion Microbiol.*, **9**, 268 (2006).
7. S. Wieschalka, B. Blombach and B. J. Eikmanns, *Appl. Microbiol. Biotechnol.*, **94**, 449 (2012).
8. J. B. McKinlay, M. Laivenieks, B. D. Schindler, A. A. McKinlay, S. Siddaramappa, J. F. Challacombe, S. R. Lowry, A. Clum, A. L. Lapidus, K. B. Burkhart, V. Harkins and C. Vieille, *Bmc Genomics*, **11**, 16 (2010).
9. M. V. Guettler, D. Rumler and M. K. Jain, *Int. J. Systematic Bacteriology*, **49**, 207 (1999).
10. Q. Li, D. Wang, Z. Song, W. Zhou, Y. Wu, J. Xing and Z. Su, *Bioresour. Technol.*, **101**, 7665 (2010).
11. Y.-l. Xi, K.-q. Chen, R. Xu, J.-h Zhang, X.-f. Bai, M Jiang, P. Wei and J.-y. Chen, *Biochem. Eng. J.*, **69**, 87 (2012).
12. M. R. Leonardo, Y. Dailly and D. P. Clark, *J. Bacteriol.*, **178**, 6013 (1996).
13. I. Martinez, G. N. Bennett and K. Y. San, *Metabolic Engineering*, **12**, 499 (2010).
14. C. Seo, H. W. Lee, A. Suresh, J. W. Yang, J. K. Jung and Y. C. Kim, *Korean J. Chem. Eng.*, **31**, 1433 (2014).
15. G. Sawers, *Current Opinion Microbiol.*, **2**, 181 (1999).
16. J. B. McKinlay, Y. Shachar-Hill, J. G. Zeikus and C. Vieille, *Metabolic Engineering*, **9**, 177 (2007).
17. J. Wang, J. F. Zhu, G. N. Bennett and K. Y. San, *Metabolic Engineering*, **13**, 328 (2011).
18. S. Alexeeva, B. de Kort, G. Sawers, K. J. Hellingwerf and M. J. T. de Mattos, *J. Bacteriol.*, **182**, 4934 (2000).
19. A. J. A. van Maris, J. M. A. Geertman, A. Vermeulen, M. K. Groo-

thuizen, A. A. Winkler, M. D. W. Piper, J. P. van Dijken and J. T. Pronk, *Appl. Environ. Microbiol.*, **70**, 159 (2004).
20. B. Zelic, S. Gostovic, K. Vuorilehto, B. Vasic-Racki and R. Takors, *Biotechnology and Bioengineering*, **85**, 638 (2004).
21. Y. Izumi, Y. Matsumura, Y. Tani and H. Yamada, *Agricultural and Biological Chemistry*, **46**, 2673 (1982).
22. H. Yanase, N. Mori, M. Masuda, K. Kita, M. Shimao and N. Kato, *J. Fermentation Bioengineering*, **73**, 287 (1992).