Appl Biochem Biotechnol (2008) 145:111–119
DOI 10.1007/s12010-007-8031-0

Succinic Acid Production from Cheese Whey using *Actinobacillus succinogenes* 130 Z

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Received: 15 May 2007 / Accepted: 27 August 2007 /
Published online: 22 September 2007

Abstract *Actinobacillus succinogenes* 130 Z was used to produce succinic acid from cheese whey in this study. At the presence of external CO_2 supply, the effects of initial cheese whey concentration, pH, and inoculum size on the succinic acid production were studied. The by-product formation during the fermentation process was also analyzed. The highest succinic acid yield of 0.57 was obtained at initial cheese whey concentration of 50 g/L, while the highest succinic acid productivity of 0.58 g h^{-1} L^{-1} was obtained at initial cheese whey concentration of 100 g/L. Increase in pH and inoculum size caused higher succinic acid yield and productivity. At the preferred fermentation condition of pH 6.8, inoculum size of 5% and initial cheese whey concentration of 50 g/L, succinic acid yield of 0.57, and productivity of 0.44 g h^{-1} L^{-1} were obtained. Acetic acid and formic acid were the main by-products throughout the fermentation run of 48 h. It is feasible to produce succinic acid using lactose from cheese whey as carbon resource by *A. succinogenes* 130 Z.

Keywords Succinic acid · Cheese whey · Lactose · Fermentation · *Actinobacillus succinogenes*

Introduction

Succinic acid, known as amber acid or butanedioic acid, is a four-carbon dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle (TCA) [1, 2]. Succinic acid and its derivative have wide industrial applications such as the feedstock of food and pharmaceutical products, as the intermediate of chemical synthesis of surfactants, detergents, green solvents, and biodegradable plastics, and also as ingredients of animal feeds to stimulate animal and

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plant growth [2–5]. Currently, most of commercial succinic acid is produced through petrochemical process, which brings environmental pollution and the concerns of sustainable development [2, 3]. The production of succinic acid by microbial fermentation is a simple and environmentally friendly process [4, 6, 7]. However, to date, biobased succinic acid is not yet competitive with petrochemical-based acid, mainly owing to high production cost [8]. There is a need to develop cost-effective conversion technology to produce succinic acid from renewable resource such as food processing waste [9–11].

Many anaerobic and facultative anaerobic microbes produce succinic acid as the fermentation end product [5]. *Actinobacillus succinogenes* 130Z, originally isolated from bovine ruminal contents, is a facultatively anaerobic, capnophilic and Gram-negative bacteria, which has been considered as the most potential succinic acid producer to produce a significant amount of succinic acid from glucose under anoxic condition [12]. This strain 130Z also showed distinctive ability to convert a broad range of carbon sources such as arabinose, cellobiose, fructose, xylose, and reduced sugar to succinate as the major end product and acetate, formate, lactate, and ethanol as the minor end products [13]. This strain has an advantage over other previously reported succinic acid producers because it can tolerate the presence of high concentration of succinic acid or its salt [14].

It was reported that *A. succinogenes* 130Z and its variant strain FZ6 produced 66.4 and 105.8 g/L of succinic acid with the yield of 0.67 and 0.8 from glucose, respectively, which indicated *A. succinogenes* had huge potential to be developed as a commercial succinic acid producer [13, 14]. Continuous and repeat-batch biofilm fermentation of succinic acid by strain 130Z demonstrated a significant increase in succinic acid productivity (7 g h^{-1} L^{-1}) and yield (86.7%) [15]. Environmental and physiological studies showed that CO_2 level and pH were the most critical factors affecting both cell growth and succinic acid formation. Increase in CO_2 supply and electron donor resulted in increase of succinic acid production and less formation of by-products such as ethanol and formate. This is most likely due to the increased PEP carboxylation to oxaloacetate rather than PEP conversion to pyruvate, where the pathway was regulated by the level of CO_2 and electron donors [16].

Whey is produced as a by-product during cheese making and as a potentially environmental pollutant due to its high biological oxygen demand (BOD) [17]. Whey consists mainly of 6 to 7% solids, of which 70 to 80% is lactose and 10 to 15% soluble proteins, lactate, and other mineral salts [11]. It can be directly used as feed additive and also has a continuing interest to be alternatively utilized as the low-cost substrate to produce value-added biochemicals such as lactic acid [17, 18]. Previous studies showed that *Actinobacillus succiniciproducens* and *Mannheimia succiniproducens* can ferment whey directly into succinic acid [11, 19]. However, the studies concerning the fermentation of succinic acid from cheese whey using A. *succinogenes* have not been reported.

The objectives of this study were to develop fermentative protocol for succinic acid production from cheese whey by *A. succinogenes* and study the effect of environmental and nutritional factors such as external CO_2 supply, pH, inoculum size, and initial whey concentration on succinic acid production.

Materials and Methods

Organism and Growth Conditions

A. succinogenes 130Z (ATCC 55618) was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in 250 mL sealed anaerobic bottles

containing 150 mL of medium with CO_2 as the gas phase, unless stated otherwise. The growth medium contained per liter deionized water: 5 g yeast extract, 3 g K_2HPO_4, 1 g NaCl, and 1 g $NaHCO_3$. The medium was autoclaved for 15 min at 121 °C in an anaerobic bottle with N_2 as headspace. Cheese whey (Davisco Foods International, Inc., Eden Prairie, MN; final concentration of 20 g/L) was separately sterilized (10 min at 110 °C) and aseptically added to the medium. pH was adjusted to 6.8 using a few drops of concentrated sterile sulfuric acid. The reduced medium was inoculated with 1.5 mL of glycerol stock and incubated at 37 °C for 20 h.

The batch fermentation was conducted in a 2.5-L fermentor (New Brunswick Scientific Co. Edison, NJ) with 1.2 L fermentation medium containing per liter: 50–100 g cheese whey, 5 g yeast extract, 10 g peptone, 3 g K_2HPO_4, 1 g NaCl, 0.02 g $CaCl_2{\cdot}H_2O$, and 0.02 g $MgCl_2{\cdot}6H_2O$. Cheese whey was separately autoclaved (10 min at 110 °C) and mixed with the nutrients in the fermentor. The pH was maintained at 6.8 using 10 N NaOH during the fermentation run, unless stated otherwise. The agitation rate and temperature were maintained at 200 rpm and 38 °C, respectively. Filter-sterile CO_2 was sparged into the medium at a constant flow rate of 0.5 vvm. The inoculum was added to the fermentor after pH, agitation, CO_2 sparging, and temperature were adjusted. Foam was controlled by adding antifoam 204 (Sigma Chemical Co., St. Louis, MO). Sample was withdrawn at an interval of 2 h during the first 8 h and an interval of 12 h during the rest of fermentation run. The fermentation experiment lasted for 48 h.

Analytical Method

The concentration of lactose and fermentation products such as succinic acid and acetic acid was determined by high-performance liquid chromatography (Waters, Milford, MA) with a KC-811 ion exclusion column and a waters 410 differential refractometer detector. The mobile phase was 0.1% H_3PO_4 solution at a flow-rate of 1 mL/min. The temperature of the detector and of the column ware maintained at 35 and 60 °C, respectively. The succinic acid yield was expressed as the amount of succinic acid produced from 1 g lactose consumed during the fermentation process.

Results and Discussions

Effect of Initial Cheese Whey Concentration

In the previous studies, it was reported that initial concentration of carbon source could influence the cell growth and succinic acid production throughout the fermentation [20]. The effect of initial cheese whey concentrations on succinic acid formation was shown in Fig. 1 Maximum succinic acid concentration of 27.9 g/L was obtained at 48 h when the initial concentration of cheese whey was 100 g/L. The succinic acid concentration increased rapidly from 6 to 24 h corresponding to the rapid consumption of lactose during this period.

Initial cheese whey concentration had a significant effect on the succinic acid yield ($P<$ 0.03) and productivity ($P<0.02$). After 48 h of fermentation, the highest succinic acid yield of 0.57 was obtained at initial cheese whey concentration of 50 g/L. The succinic acid productivity increased from 0.44 to 0.58 g h^{-1} L^{-1} when the initial whey concentration increased from 50 to 100 g/L (Fig. 2). The highest succinic acid productivity of 0.95 g h^{-1} L^{-1} was obtained at 24 h with the initial whey concentration of 75 g/L, while the productivity

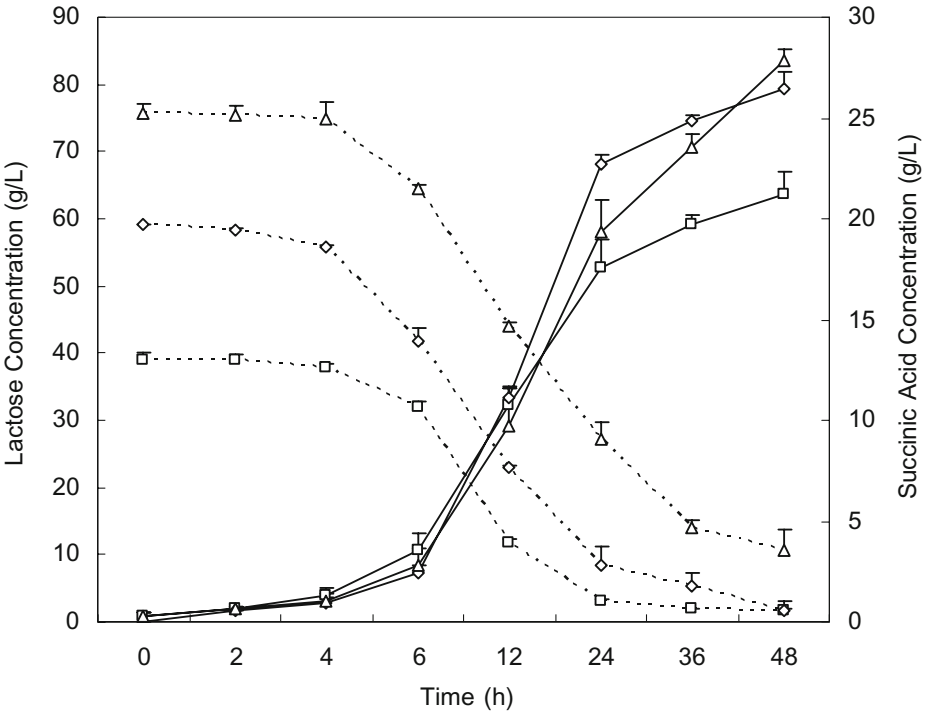

Fig. 1 Effect of initial cheese whey concentration on lactose and succinic acid concentration (pH 6.8, inoculum size of 5%). *Solid line and dash line* represent the succinic acid concentration and the lactose concentration, respectively. *Symbols* represent different cheese whey concentrations of 50 (*empty squares*), 75 (*empty diamonds*), and 100 g/L (*empty triangles*)

at 48 h was 0.44 g h^{-1} L^{-1} (Fig. 2). The rapid increase in succinic acid concentration from 12 to 24 h led to the higher productivity at 24 h. In our study, it was demonstrated that strain 130Z could directly ferment lactose from cheese whey with less single sugar such as galactose and glucose produced due to the degradation of lactose (data no shown).

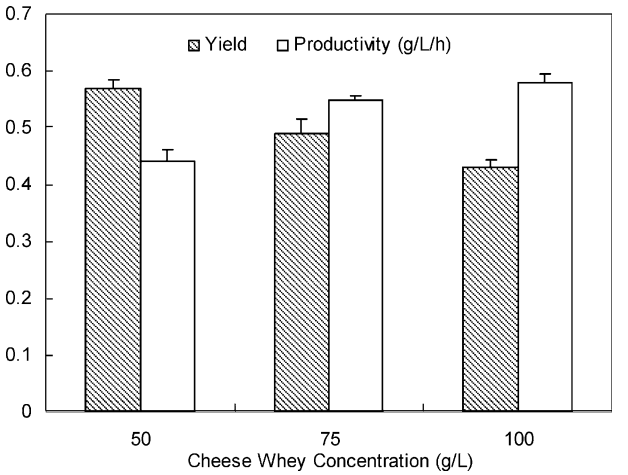

Fig. 2 Effect of initial cheese whey concentration on the succinic acid yield and productivity (pH 6.8, inoculum size of 5%, fermentation time of 48 h)

Effect of the pH

The pH is an important environmental factor that affects cell growth and CO_2/HCO_3^- ratio which played a crucial role in the fermentative succinic acid production. Samuelov et al. [11] reported that low pH resulted in increase of the activity of the key enzymes of the PEP carboxykinase pathways, which led to the flux of PEP towards more succinic acid formation. However, low pH also had adverse effect on the cell growth [16]. The optimal pH or range for succinic acid production by anaerobic or facultatively anaerobic microorganism such as *Bacteroides fragilis*, *A. succiniciproducens, M. succiniciproducens* MBEL 55E, *Enterococcus facalis,* and *A. succinogenes* was 7.0, 6.8, 6.0–7.5, 7–8, and 6.0–7.4, respectively [7, 11, 14, 16, 21, 22].

The effect of pH on succinic acid production using cheese whey as carbon source by *A. succinogenes* 130 Z was shown in Fig. 3. The concentration of succinic acid increased from 19.1 to 21.2 g/L with pH increase from 6.2 to 6.8, while the succinic acid concentration at pH 6.8 and 7.2 were very close. The succinic acid yield and productivity at pH 6.8 and 7.2 were also very close, while that at pH 6.2 were much lower (Fig. 4). However, the effects of pH ranging from 6.2 to 7.2 on the succinic acid yield ($P>0.13$) and productivity ($P>0.35$) were not significant. Although similar succinic acid productivity and yield were obtained at pH 6.8 and 7.2, more alkaline is needed to maintain a higher pH during fermentation run. In this study, the preferred pH for succinic acid production from cheese whey was 6.8.

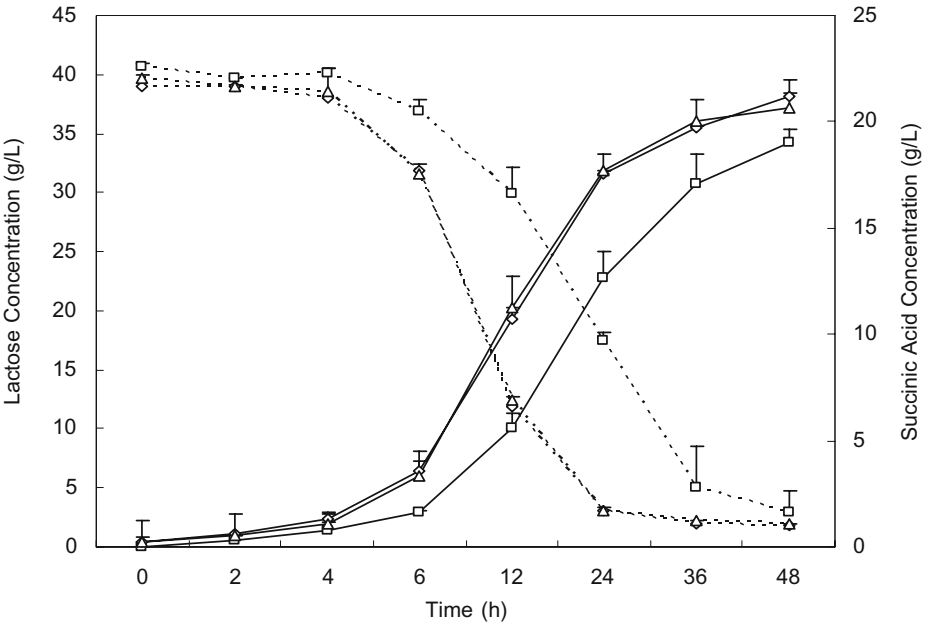

Fig. 3 Effect of pH value on lactose and succinic acid concentration (initial cheese whey concentration of 50 g/L, inoculum size of 5%). *Solid line* and *dash line* represent the succinic acid concentration and the lactose concentration, respectively. Symbols are the pH values of 6.2 (*empty squares*), 6.8 (empty diamonds), 7.2 (*empty triangles*)

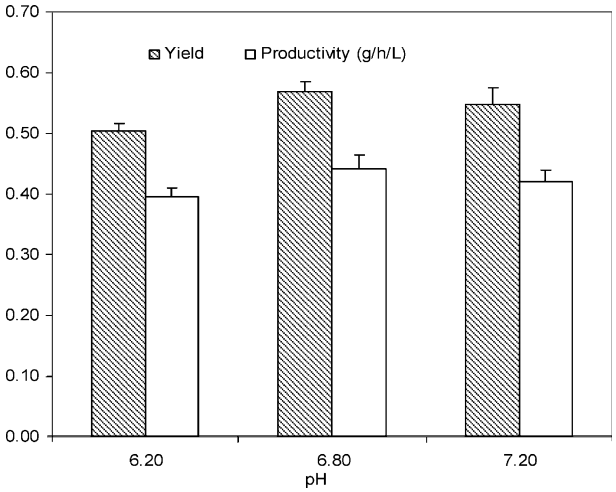

Fig. 4 Effect of pH on the succinic acid yield and productivity (initial cheese whey concentration of 50 g/L, inoculum size of 5%, total fermentation time of 48 h)

Effect of Inoculum Size

The effect of inoculum size on succinic acid production is shown in Fig. 5. Higher succinic acid concentration was observed at the inoculum size of 10% than that at 2 and 5%

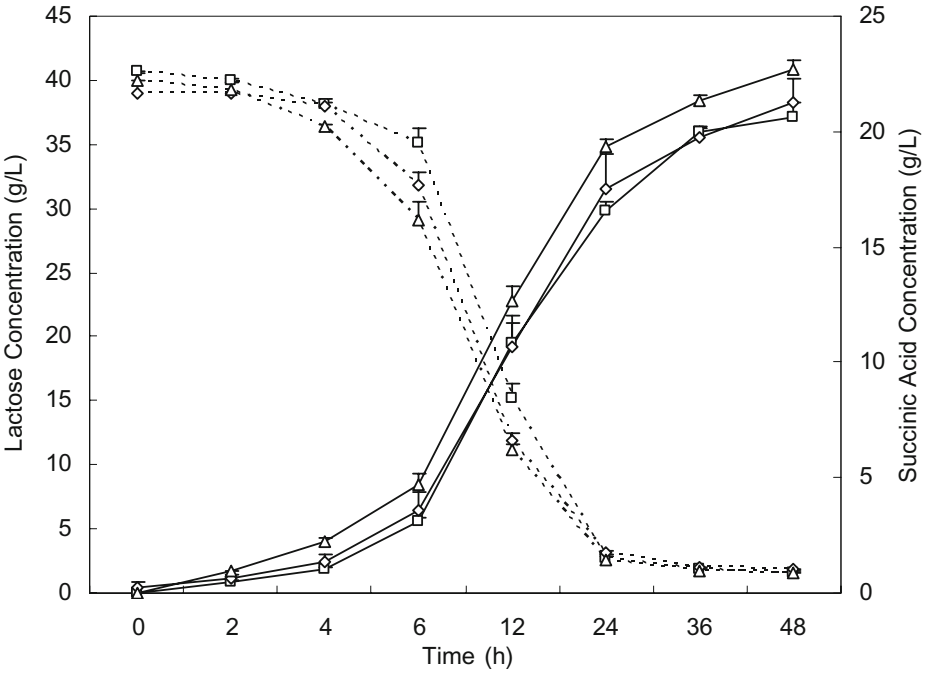

Fig. 5 Effect of inoculum size on lactose and succinic acid concentration (initial cheese whey concentration of 50 g/L, pH 6.8). *Solid line* and *dash line* represent the succinic acid concentration and the lactose concentration, respectively. *Symbols* are the inoculum sizes of 2 (*empty squares*), 5 (*empty diamonds*), 10% (*empty triangles*)

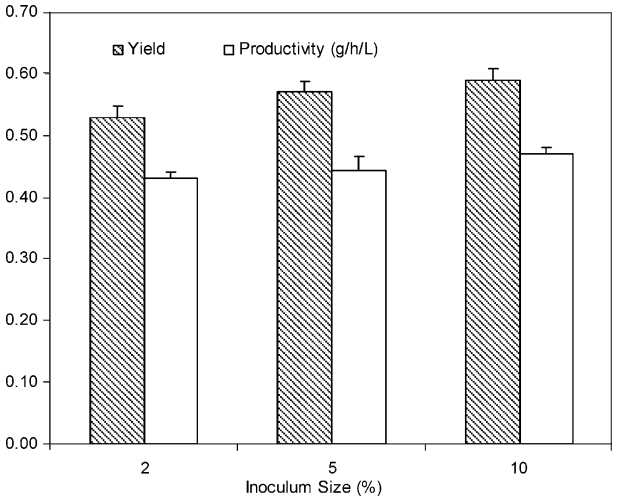

Fig. 6 Effect of inoculum size on the succinic acid yield and productivity (initial cheese whey concentration of 50 g/L, pH of 6.8, total fermentation time of 48 h)

throughout the fermentation run. The reason could be that the increase in the inoculum density shortened the lag period and increased the final cell concentration, which resulted in the less fermentation time required to reach the maximum succinic acid concentration [22]. It can be seen from Fig. 6 that maximum succinic acid yield (0.59) and productivity (0.47 g h^{-1}

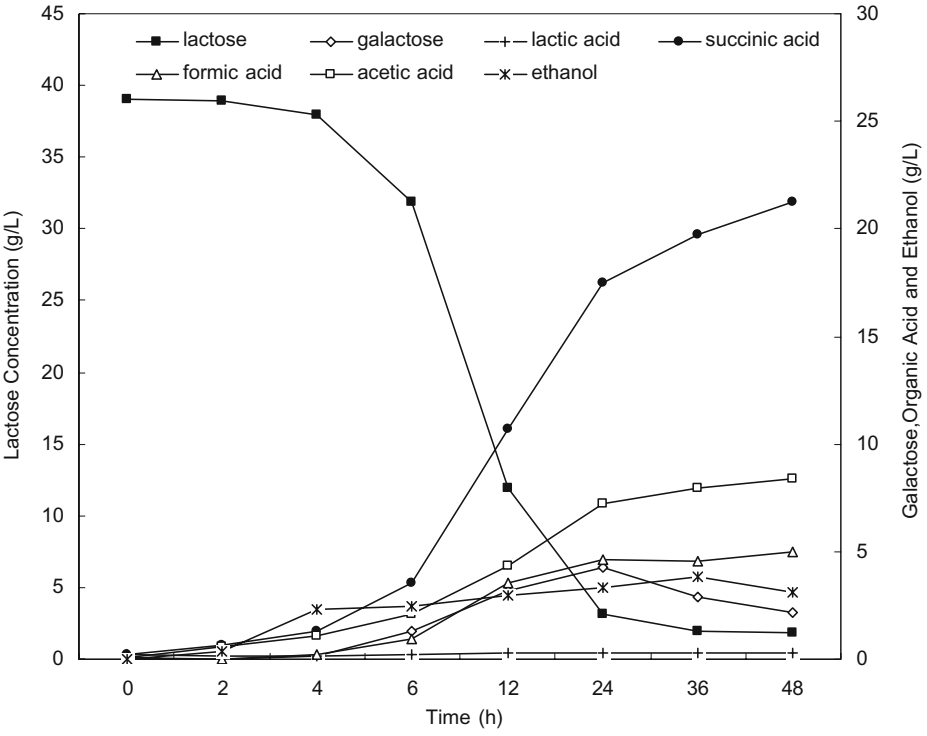

Fig. 7 Distribution of end products during the fermentative succinic acid production (initial cheese whey concentration of 50 g/L, pH of 6.8, and inoculum size of 5%)

L^{-1}) were obtained at inoculum size of 10%. However, the effects of inoculum sizes on succinic acid yield ($P>0.11$) and productivity ($P>0.36$) were not significant.

The Production of By-Products

Under anoxic condition of succinic acid production, phoshpoenolpyruvate (PEP), one of the central intermediates during mixed acid fermentation, is converted by two enzymes in *A. succinogenes* 130Z named PEP carboxykinase (PPCK) and pyruvate kinase [16, 23]. PPCK is a CO_2-fixing enzyme that converts PEP to oxaloacetate toward the flux to the formation of succinic acid [20, 23]. Pyruvate kinase converts PEP to pyruvate, which is consequently converted to end fermentation by-products such as acetic acid, formic acid, and ethanol [16].

The end fermentation products at initial cheese whey concentration of 50 g/L, pH 6.8 and inoculum size of 5% under non-limiting CO_2 supply (0.5 vvm CO_2 sparging) are shown in Fig. 7. Acetic acid and formic acid were the main by-products, and there was no observation of lactic acid production throughout the fermentation run. The concentration of acetic acid increased more rapidly than that of other by-products along with the dramatic increase of succinic acid concentration from 6 to 24 h. No further increase of formic acid was observed after 24 h, while there was a slight increase in the concentration of acetic acid. The galactose concentration resulting from lactose degradation slightly decreased after 24 h possibly due to the fermentation of galactose by *A. succinogenes* 130Z at the low total carbohydrate concentration.

Conclusion

This study shows that *A. succinogenes* 130Z can effectively convert cheese whey to succinic acid. Therefore, cheese whey, waste stream from dairy industry, can be used in the cost-effective fermentative production of succinic acid.

Higher succinic acid productivity and lower yield were obtained when the initial cheese whey concentration increased from 50 to 100 g/L. Increase in succinic acid productivity and yield was also obtained with increased pH range from 6.2 to 7.2. The highest succinic acid yield of 0.57 was obtained at pH 6.8. Higher succinic acid yield and productivity were obtained when the inoculum size increased from 2 to 10%, but the effect was not significant. Under non-limiting CO_2 supply, acetic acid and formic acid were the main by-products during the succinic acid production from cheese whey using *A. succinogenes* 130Z. Further studies need to be conducted to determine the optimized fermentation parameters for maximum succinic acid production from cheese whey using *A. succinogenes* 130Z.

Acknowledgements The authors thank Michele R. Mims for the assistance in sample analysis. Financial support from USDA CSREES Evans-Allen Program is also greatly appreciated.

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