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Succinic Acid Production by *Anaerobiospirillum succiniciproducens* ATCC 29305 Growing on Galactose, Galactose/Glucose, and Galactose/Lactose

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Succinic acid-producing *Anaerobiospirillum succiniciproducens* was anaerobically grown on galactose, galactose/glucose, or galactose/lactose in order to study its galactose fermentation. Unlike a previous report, *A. succiniciproducens* was found to efficiently metabolize galactose as the sole carbon source at a rate of 2.4 g/g-DCW/h and produced succinic acid with as high a yield of 87% as with using glucose. When glucose and galactose were present, *A. succiniciproducens* metabolized both sugars simultaneously. Furthermore, when lactose and galactose coexisted, lactose did not inhibit the galactose fermentation of *A. succiniciproducens*. Therefore, co-utilization of galactose and other sugars can improve the productivity and economy of bio-based succinic acid processes.

Keywords: *Anaerobiospirillum succiniciproducens*, succinic acid, galactose, mixed sugar fermentation

Succinic acid, a dicarboxylic acid, has been considered as one of the biochemicals of commercial interest because it can be used for the manufacture of synthetic resins and biodegradable polymers and as an intermediate for the synthesis of various chemicals [22, 31]. Even though, to date, most of succinic acid has been produced by chemical processes, biological production mainly exploiting succinic acid-producing bacteria has attracted great interest as an alternative route for succinic acid [13]. In the view of metabolism and physiology, succinic acid has a dual role as an intermediate of the TCA cycle and as fermentative end-products of several anaerobic and facultative microorganisms [7, 29]. The well-known succinic acid-producing anaerobic and facultative microorganisms are *Anaerobiospirillum succiniciproducens* [5, 14], *Actinobacillus succinogenes* [8], and *Mannheimia succiniciproducens* [11, 12, 15].

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Among them, the obligate anaerobe *A. succiniciproducens* has been considered as one of the best succinic acid producers studied so far because it produces succinic acid at a high yield, uses a wide spectrum of carbohydrates as a carbon and energy source, and forms less by-products [5, 23]. This promising fermentation capability of *A. succiniciproducens* has initiated studies on the physiological response of the cells to various environmental or cultural conditions such as CO_2-HCO_3^- levels on cell growth [18, 28] and medium compositions [19]. Currently, many biotechnological processes utilize agricultural, food, or oil industries wastes, in particular mixed sugars, as cheap nutrient sources for the productions of biocommodity chemicals [22]. Succinic acid, one of the promising biocommodities, could be efficiently produced by culturing *A. succiniciproducens* on cheap wastes nutrients such as wood hydrolysates [16] and whey (lactose) [17]. Interestingly, in our previous study, *A. succiniciproducens* was found to take up a small amount of galactose from a culture medium during fermentation of lactose and whey [17]. This galactose consumption, however, is contrary to the study of Davis *et al.* [5], in which *A. succiniciproducens* was unable to metabolize galactose as a carbon source.

Microbial utilization of galactose is valuable in industrial biotechnology processess where renewable or by-product materials such as lignocellulose or beet molasses are mainly used as nutrient sources, since they contain galactose in the form of hemicellulose and raffinose, respectively [26]. Furthermore, commonly used industrial carbon sources are composed of a mixture of sugars, one of which frequently represses the other sugars and thus sugars are utilized sequentially [27]. Thus, a simultaneous utilization of mixed sugars can improve the productivity of biotechnology processes by shortening fermentation times [25]. Therefore, it is of great importance to elucidate the galactose fermentation of *A. succiniciproducens* and to examine the kinetics of *A. succiniciproducens* growing on galactose only and on mixed sugars.

In this study we report on the galactose fermentation of *A. succiniciproducens* ATCC 29305 under controlled conditions. Furthermore, the effect of glucose and lactose on galactose metabolism is also reported. *Anaerobiospirillum succiniciproducens* (ATCC 29305) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Precultures were grown in sealed anaerobic bottles (200 ml) containing 100 ml of minimal salts medium1 (AnS1) containing 5 g/l glucose or galactose as a carbon source, 2.5 g/l polypeptone, and 2.5 g/l yeast extract, with CO_2 as the gas phase. The AnS1 medium contained per liter: 3 g K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, and 1 g Na_2CO_3. The medium was heat sterilized (15 min at 121°C) in an anaerobic bottle with nitrogen headspace. To the sterile medium, concentrated H_2SO_4 was added to adjust the pH to 6.5. The nitrogen headspace was replaced by CO_2, and $Na_2S \cdot 9H_2O$ was added to a final concentration of 1 mg/l to ensure strict anaerobic condition. After 15 min, the reduced medium was inoculated with 2.5 ml of glycerol stock culture and incubated at 39°C for 18 h in an anaerobic chamber (Forma Sci., U.S.A.).

For anaerobic flask experiments, exponentially growing precultures grown on glucose or galactose, washed anaerobically 2 times with a sugar-deficient medium, were used to inoculate sealed anaerobic bottles containing 100 ml of a minimal salts medium2 (AnS2). The AnS2 medium contained per liter: 3 g K_2HPO_4, 1 g NaCl, 5 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, and 10 or 20 g $MgCO_3$. To the AnS2 medium, 10 g/l galactose was added and grown anaerobically at 39°C.

Anaerobic batch cultures were performed at 39°C in a jar fermenter (2.5 l; Korea Fermenter Company, Incheon, Korea) containing 1 l of AnS2 medium containing galactose, a mixture of galactose and glucose, or galactose and lactose as carbon sources. The pH was controlled at 6.5 using 2 M Na_2CO_3. Foaming was controlled by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO, U.S.A.). The CO_2 sparging rate and agitation speed were controlled at 0.25 vvm and 200 rpm, respectively. All chemicals used were of reagent grade and were obtained from Junsei Chemical Co. (Tokyo, Japan), Difco Laboratories (Detroit, MI, U.S.A.), or Sigma Chemical Co. Gas was scrubbed free of oxygen by passing through a gas purifier (P.J. Cobert Associates, Inc., St. Louis, MO, U.S.A.).

The concentrations of galactose, glucose, lactose, succinic acid, and acetic acid were measured by high-performance liquid chromatography (Hitachi L-3300 RI monitor, L-4200 UV-VIS detector, D2500 chromato-integrator; Tokyo, Japan) equipped with an ion-exchange column (Aminex HPX-87H, 300 mm×7.8 mm; Hercules, CA, U.S.A.) using 0.012 N H_2SO_4 as a mobile phase. Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer (Ultrospec3000; Pharmacia Biotech, Sweden). Dry cell weight (DCW) was calculated from a standard curve relating the OD_{660} to dry cell weight (1 OD_{660}=0.33 g-DCW/l). Succinic acid yield was defined as the gram amount of succinic acid produced from one gram of carbohydrate (or equivalent), and was expressed as a percentage. Theoretically, 1 mole of succinic acid is fermentatively formed by incorporating 1 mole of CO_2 into 1/2 mole of glucose [18].

The activity of β-galactosidase was measured with *o*-nitrophenyl-β-D-galactopyranoside as a substrate in Z-buffer, as described by Miller [24]. The unit for the volumetric β-galactosidase activity was defined as μmole *o*-nitrophenol formed per milliliter of culture per minute at 28°C, pH 7.0. All assays were carried out in triplicates.

It is well demonstrated that many microorganisms tend to be affected by catabolic repression when mixed sugars are present and by preculture conditions [1, 4, 6, 9]. This is true for the lactose fermentation of *A. succiniciproducens* [17]. However, unlike the lactose fermentation, *A. succiniciproducens* pregrown on glucose rapidly metabolized galactose and grew well as if pregrown on galactose (data not shown). Therefore, to better understand the galactose fermentation of *A. succiniciproducens*, the cells were anaerobically grown on 17.5 g/l of galactose under a well-controlled condition. *A. succiniciproducens* completely metabolized galactose at the maximum specific consumption rate of 2.4 g/g-DCW/h in 10.5 h and grew rapidly to reach 1.1 g-DCW/l (Fig. 1). Succinic acid of 15.3 g/l was produced with a conversion yield of 87%, and also acetic acid of 4.6 g/l, a by-product, was produced in 10.5 h. The gram ratio of succinic acid/acetic acid (S/A) was 3.3:1, which was slightly lower than those

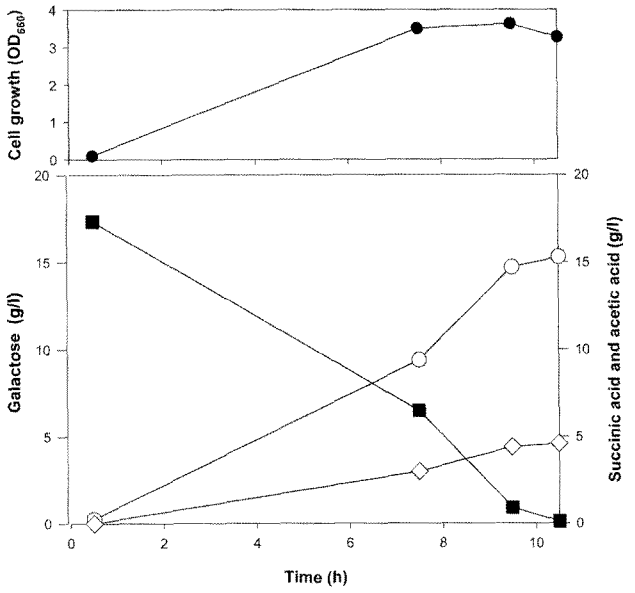

Fig. 1. Batch fermentation of galactose as a carbon source by *A. succiniciproducens*.
Symbols are cell concentration (●), galactose (■), succinic acid (○), and acetic acid (◇).

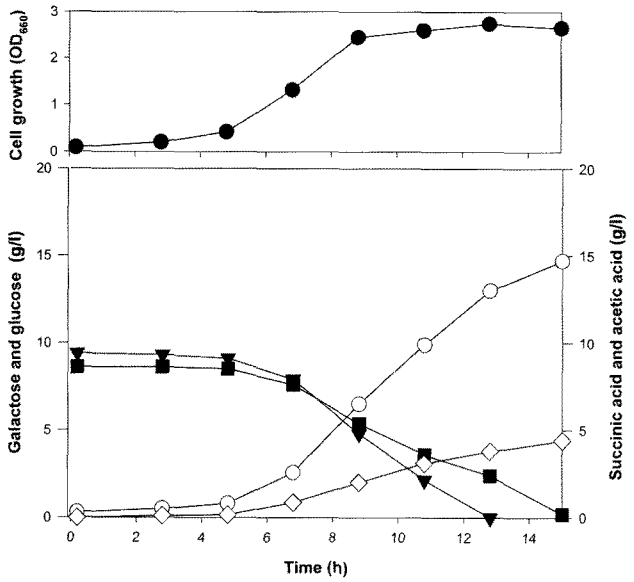

Fig. 2. Mixed sugar fermentation of galactose and glucose by *A. succiniciproducens*.
Symbols are cell growth (●), galactose (■), glucose (▼), succinic acid (○), and acetic acid (◇).

(3.7–4.1) obtained on glucose [19] or lactose [17]. It is not clear why galactose consumption decreases slightly the S/A ratio, but one possible reason might be the fast growth of *A. succiniciproducens* on galactose. *A. succiniciproducens* tends to produce more acetate when it grows faster [18]. There were no other metabolites, such as lactic acid, detectable in the culture media. This result suggests that galactose fermentation would not significantly influence succinic acid and acetic acid formation of *A. succiniciproducens*, unlike glycerol [20]. This efficient utilization of galactose without a significant physiological/metabolic change can make bio-based succinic acid production using *A. succiniciproducens* more feasible and economical. Many lactic acid bacteria such as *Lactococcus lactis* ssp. *cremoris* [2] and *Streptococcus salivarius* ssp. *thermophilus* [10] utilize the glucose moiety of lactose, but not the galactose moiety. In some cases, remaining galactose even exerts feedback inhibition on the metabolism of lactose or glucose in bacteria [3].

As mentioned above, most industrial media contain mixed sugars, which can be sequentially or simultaneously metabolized by the exploited microorganisms. Therefore, the efficient and simultaneous utilization of mixed sugars is one of the important factors affecting the overall productivity of biotechnology processess. First, we tested a mixture of glucose (9.4 g/l) and galactose (8.6 g/l) as carbon sources. As shown in Fig. 2, *A. succiniciproducens* did not show a diauxic growth pattern and simultaneously consumed glucose and galactose at the maximum specific consumption rates of 3.6 g/g-DCW/h for glucose and 2.4 g/g-DCW/h for galactose. These maximum consumption rates are comparable to those obtained by galactose or glucose as the sole carbon source (Table 1), indicating that the coexistence of glucose may not inhibit transport and catabolism of galactose, and vice versa. The co-fermentation of glucose and galactose did not significantly affect succinic acid formation and yield (14.7 g/l and 87%) or acetic acid formation (4.3 g/l). This indicates that *A. succiniciproducens* can grow on glucose and galactose without any catabolic suppression [21, 30]. It might be that *A. succiniciproducens* uses a constitutive catabolic system for galactose as in glucose.

Next, the mixed sugars lactose and galactose were examined because whey(lactose)-based medium is one of the preferred industrial media and galactose is frequently detected in the culture medium [17]. *A. succiniciproducens* pregrown on lactose consumed galactose first at a specific consumption rate of 2.3 g/g-DCW/h, and after galactose was depleted lactose started to be utilized at a rate of 0.43 g/g-DCW/h (Fig. 3). This sequential sugar consumption pattern was the same as in the co-fermentations of glucose and lactose [17]. Interestingly, whereas the coexistence of lactose did not affect the galactose fermentation of *A. succiniciproducens*, the presence of galactose inhibited the lactose fermentation. As shown in Fig. 3. *A. succiniciproducens* started to consume lactose with a high β-galactosidase activity as galactose started to be depleted. As with galactose and glucose, the co-fermentation of galactose and lactose did not affect carbon flux directed toward succinic acid and acetic acid formations.

In conclusion, we show in this study that *A. succiniciproducens* efficiently utilized galactose as the sole carbon source at a high consumption rate. Furthermore,

Table 1. Data from the anaerobic batch cultivation of *A. succiniciproducens* on carbon sources.

Carbon source	Maximum species growth rate (h^{-1})	Succinic acid yield (g-succinic acid/g-substrates)	Maximum sugar consumption rate (g-substrate/g-DCW/h)	Reference
Glucose	0.55	88	Glucose: 3.8	[19]
Lactose	0.50	87	Lactose: 1.02	[17]
Lactose+glucose	0.51	86	Lactose: 0.35; Glucose: 3.7	[17]
Galactose	0.56	87	Galactose: 2.4	This study
Galactose+glucose	0.56	87	Galactose: 2.4; Glucose: 3.6	This study
Galactose+lactose	0.55	88	Galactose: 2.3; Lactose: 0.43	This study

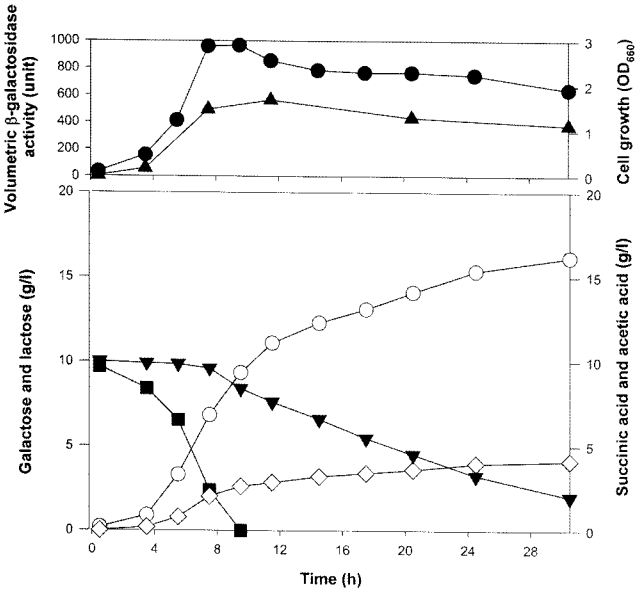

Fig. 3. Mixed sugar fermentation of galactose and lactose by *A. succiniciproducens*.
Symbols are cell growth (●), volumetric β-galactosidase activity (▲), galactose (□), lactose (▼), succinic acid (○), and acetic acid (◇).

A. succiniciproducens simultaneously utilized galactose and glucose without significant physiological/metabolic changes. This simultaneous co-utilization of mixed sugars can improve the performance of bio-based succinic acid processes.

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