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# Continuous succinic acid fermentation by *Actinobacillus succinogenes*

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ABSTRACT

Fermentations were performed in an external recycle bioreactor using $CO_2$ and D-glucose at feed concentrations of 20 and 40 g $L^{-1}$. Severe biofilm formation prevented kinetic analysis of suspended cell ('chemostat') fermentation, while perlite packing enhanced the volumetric productivity by increasing the amount of immobilised cells. The highest productivity of 6.35 g $L^{-1}$ $h^{-1}$ was achieved at a dilution rate of 0.56 $h^{-1}$. A constant succinic acid yield of 0.69 ± 0.02 g/(g of glucose consumed) was obtained and found to be independent of the dilution rate, transient state and extent of biofilm build-up – approximately 56% of the carbon that formed phosphoenolpyruvate ended up as succinate. Byproduct analysis indicated that pyruvate oxidation proceeded solely via the formate-lyase pathway. Cell growth and corresponding biofilm formation were rapid at dilution rates higher than 0.35 $h^{-1}$ when the product concentrations were low (succinic acid < 10 g $L^{-1}$), while minimal growth was observed at succinic acid concentrations above this threshold.



## 1. Introduction

In the past decade butanedioic acid or succinic acid (SA) has established itself as a forerunner in biorefinery platform chemicals. Four carbon dicarboxylic acids were already identified in the US Department of Energy's list (2004) of potential large-scale biomass-derived chemicals and in an updated report SA features as a standalone molecule [1,2]. It is therefore no surprise that commercial bioproduction of SA by companies such as BioAmber, Reverdia, Myriant Technologies and a joint venture between BASF and DSM-Purac is either already happening or in the construction phase. These four companies plan to produce SA in excess of 150,000 tons per annum by the end of 2015 [3,4].

Reactor design and operation of SA fermentation is likely to become more important with the prospect of bulk scale production on the horizon. Most of the current focus is on the development of the microorganism, with batch operation being the preferred mode of operation. Continuous production of SA is likely to outperform batch processing, especially when considering the projections of future processing quantities. In this regard the number of studies on continuous SA producing cultures is limited. The most documented SA producers are wild strains of *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and various recombinant strains of *Escherichia coli* [5,6]. These bacteria, in addition to recombinant *Corynebacterium glutamicum*, have been identified by McKinlay et al. [7] as the most promising SA producers. The wild strains are anaerobes that produce SA naturally as a major catabolic product via the phosphoenolpyruvate carboxykinase pathway [8].Modified *E. coli* have been engineered to either copy the above-mentioned pathway with less byproduct formation [9] or produce SA aerobically via an interrupted TCA cycle [10]. The theoretical maximum yield is 1.71 mol SA per mol glucose (1.12 g $g^{-1}$) when redox requirements are considered and biomass formation is ignored.

Only the wild SA-producing strains (*A. succinogenes*, *M. succiniciproducens* and *A. succiniciproducens*) have been studied under continuous conditions. With the exception of the work done by Urbance et al. [11] and Meynial-Salles et al. [12], all continuous SA work was done at the Korean Institute of Advanced Technology (KAIST) where *M. succiniciproducens* was first isolated [13–19]. Apart from normal suspended cell systems ('chemostat'), membrane systems with cell recycle as well as biofilm reactors were used in an attempt to enhance productivity [11–13,17]. Particular success was achieved by Meynial-Salles et al. [12] with *A. succiniciproducens* where a very high productivity of 14.8 g $L^{-1}$ $h^{-1}$ was obtained with a SA yield of 0.83 g $g^{-1}$. In addition to the cell recycle membrane reactor, a system was presented where an electrodialysis unit was utilised in conjunction with the reactor to remove organic acids in situ and thereby increase the final product titer up to 80 g $L^{-1}$ (at a productivity of 10.4 g $L^{-1}$ $h^{-1}$). Urbance et al. [11] also reported high productivities (up to 8.8 g $L^{-1}$ $h^{-1}$) by *A. succinogenes* where a special polypropylene composite support was used to enhance immobilisation. The results are, however, scattered with

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**Nomenclature**

| | |
|---|---|
| $C$ | concentration/titer ($g\,L^{-1}$) |
| $D$ | dilution rate ($h^{-1}$) |
| $F$ | molar flowrate ($mol\,h^{-1}$) |
| $M$ | molar mass ($g\,mol^{-1}$) |
| $P$ | volumetric productivity ($g\,L^{-1}\,h^{-1}$) |
| $V$ | volume of reactor (L) |
| $Y$ | yield ($g\,g^{-1}$) |

*Subscripts*

| | |
|---|---|
| S | substrate |
| SA | succinic acid |

low SA yields at high productivities. Also, no details on byproducts are provided. The chemostat data on *A. succiniciproducens* by Lee et al. [19] present a thorough set of data for two different glucose feed concentrations from which a consistently high yield over a broad range of dilution rates was obtained. A summary of continuous succinic acid fermentation studies is given in Table 1.

This work presents the third continuous study on *A. succinogenes* and includes a significant extension to the studies by Urbance et al. [11] and Kim et al. [13]. Emphasis is placed on the transient behaviour of the system, byproduct distribution and the potential advantages of utilising the immobilisation capabilities of *A. succinogenes*.

## 2. Materials and methods

### 2.1. Microorganism

*A. succinogenes* 130Z (DSM 22257 or ATCC 55618) was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ). Vials containing treated beads in a cryopreservative solution were used to store culture samples at −75 °C. The inoculum was incubated at 38 °C and 100 rpm over a period of 20–24 h in 30 mL sealed bottles containing 15 mL tryptone soy broth (TSB).

### 2.2. Media composition

The medium was based on the formulations tested by Urbance et al. [20]. Chemicals were obtained from Merck KgaA (Darmstadt, Germany) unless indicated otherwise. The concentrations of the components in the medium were: 6 $g\,L^{-1}$ yeast extract, 10 $g\,L^{-1}$ corn steep liquor (Sigma–Aldrich, St. Louis, USA), 0.3 $g\,L^{-1}$ $Na_2HPO_4$, 1.4 $g\,L^{-1}$ $NaH_2PO_4$, 1.4 $g\,L^{-1}$ sodium acetate, 1 $g\,L^{-1}$ NaCl, 1.5 $g\,L^{-1}$ $K_2HPO_4$, 0.2 $g\,L^{-1}$ $MgCl_2{\cdot}6H_2O$, 0.23 $g\,L^{-1}$ $CaCl_2{\cdot}2H_2O$, 0.05 $g\,L^{-1}$ antifoam A (Sigma–Aldrich, St. Louis, USA) and 20 or 40 $g\,L^{-1}$ D-glucose. $CO_2$ (g) (African Oxygen, Johannesburg, South Africa) was used as the inorganic carbon source and was sparged through the reactor at approximately 0.05 vvm.

### 2.3. Fermentation

The bioreactor setup with an external recycle used for agitation is illustrated in Fig. 1. Each reservoir was fitted with a 0.2 μm PTFE membrane filter (Midisart 2000 filters from Sartorius, Göttingen, Germany). The total open volume of fermentations was 156 mL and included the 'bioreactor' represented in Fig. 1 and the recycle line. The bioreactor consisted of an aluminium top and bottom section and a glass tube with a length of 115 mm and an inner diameter of 37.5 mm. The bottom section contained one entry/exit point for fermentation broth and also transferred heat from the hotplate to the broth. The top section contained an aluminium sheath that acted as a thermowell for the thermocouple and two additional entry/exit points for the broth. The connected, closed setups indicated by the dashed lines in Fig. 1 were sterilised in an autoclave at 121 °C for 40 min. The medium containing yeast extract, corn steep liquor and salts was autoclaved separately from the glucose solution and then mixed aseptically. The reactor was filled and operated at a stable temperature and pH before it was seeded with approximately 8–10 mL of the inoculum. 10 M non-sterile potassium hydroxide (KOH) was used to control the pH at 6.80 ± 0.05. During startup the fermenter was operated at the lowest dilution rate that prevented the reactor from emptying through froth entrainment. This was done to approach batch conditions to allow for initial accumulation of biomass.

The same reactor setup was employed for the biofilm fermentations. Genulite™ Groperl (Infigro Natural Technologies, Johannesburg, South Africa) particles with equivalent diameters of 2–4 mm were added to the reactor to provided increased area for cell attachment. Groperl particles are expanded perlite particles consisting of amorphous volcanic glass. Due to geometric constraints, only 40% of the reactor's open volume could be filled with packing.

### 2.4. Analytical methods

Glucose, ethanol and organic acid concentrations were determined by using high-performance liquid chromatography. An Agilent 1260 Infinity HPLC (Agilent Technologies, USA), equipped with an RI detector and a 300 mm × 7.8 mm Aminex HPX-87H ion-exchange column (Bio-Rad Laboratories, USA) was used. The mobile phase (0.3 mL $L^{-1}$ $H_2SO_4$) was fed at a flowrate of 0.6 mL $min^{-1}$ with a column temperature of 60 °C. Dry cell weight (DCW) was determined from 4.5 mL samples centrifuged at 12 100 g for 5 min.Cell

**Table 1**
Summary of continuous succinic acid fermentation studies.

| Microorganism | Substrate ($g\,L^{-1}$) | $D$ ($h^{-1}$) | Reactor type | Max $P_{SA}$ ($g\,L^{-1}\,h^{-1}$) | $Y_{SA/S}$ ($g\,g^{-1}$) | Max $C_{SA}$ ($g\,L^{-1}$) | References |
|---|---|---|---|---|---|---|---|
| *A. succinogenes* 130Z | Glucose: 20 | 0.2–1.2 | Suspended cell, biofilm | 8.8 | 0.27–0.73 | 10.4 | [11] |
| *A. succinogenes* 130Z | Glucose: 60 | 0.2–0.5 | Cell recycle | 6.63 | 0.50–0.59 | 18.6 | [13] |
| *A. succiniciproducens* ATCC No. 29305 | Lactose: 45 | 0.085–0.15 | Suspended cell | 3.0 | 0.62–0.72 | 24.0 | [29] |
| *A. succiniciproducens* ATCC No. 29305 | Lactose: 20 | 0.03–0.14 | Suspended cell | 1.4 | 0.81–0.94 | 14.0 | [16] |
| *A. succiniciproducens* ATCC No. 53488 | Glucose: 20 | 0.19–0.93 | Cell recycle | 14.8 | 0.74–0.83 | 16.5 | [17] |
| *A. succiniciproducens* ATCC No. 29305 | Glucose: 19, 38 | 0.032–0.63 | Suspended cell | 6.5 | 0.73–0.82 | 29.6 | [18] |
| *A. succiniciproducens* ATCC No. 29305 | Glycerol: 10.7, 11, 11.3 | 0.022–0.25 | Suspended cell | 2.2 | 1.23–1.50 | 16.1 | [19] |
| *B. succiniciproducens* DD1 | Glycerol: 5.1 | 0.004–0.018 | Suspended cell | 0.0094 | 0.71–1.02 | 5.2 | [30] |
| *M. succiniciproducens* MBEL55E | Lactose: 21 | 0.1–0.7 | Suspended cell | 3.9 | 0.63–0.69 | 10.3 | [15] |
| *M. succiniciproducens* MBEL55E | Glucose and xylose: 18, 7 | 0.1–0.7 | Suspended cell | 3.2 | 0.34–0.61 | 14.1 | [14] |
| *M. succiniciproducens* LPK7 | Glucose: 9, 18 | 0.1–0.3 | Suspended cell | 1.77 | 0.10–0.71 | 12.9 | [31] |
| *M. succiniciproducens* MBEL55E | Glucose: 20 | 0.1–0.3 | Cell recycle | 2.85 | 0.48–0.64 | 12.8 | [13] |