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The effect of carbon dioxide availability on succinic acid production with biofilms of *Actinobacillus succinogenes*

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ABSTRACT

Carbon dioxide serves as a co-substrate in succinic acid (SA) production by *Actinobacillus succinogenes* making it an important consideration in fermentation optimisation. In the current study, the availability of CO_2 to the cell, as the dissolved CO_2 concentration in the fermentation broth (C_{CO_2}), is shown to define three distinct steady-state regimes. At C_{CO_2} values between 8.4 mM (±36.8% saturation) and saturation (22.8 mM), there is no evidence of CO_2 limiting SA productivity and flux to SA is constant. As C_{CO_2} is decreased, an upper C_{CO_2} threshold (±36.8% saturation; 8.4 mM) is reached where metabolic flux distributions remain constant but SA productivity and substrate uptake start to decline with decreasing C_{CO_2} levels. A further decrease in C_{CO_2} leads to a lower C_{CO_2} threshold (±17.1% saturation, 3.9 mM) where SA productivity continues to decrease with a concomitant shift in carbon flux away from SA towards C_3 fermentative pathways including ethanol. Since SA production is not limited at relatively low C_{CO_2} values (±36.8% saturation), adequate CO_2 supply to the fermenter can be achieved without requiring major CO_2 sparging schemes which is favourable from an industrial processing perspective.

1. Introduction

Succinic acid has been identified as a top value-added [1] and bulk [2] chemical derived from biomass. This stems from both its presence in the tricarboxylic acid cycle which makes biological production plausible, as well as its potential to serve as a substitute and precursor for a number of petrochemicals, thereby augmenting the established market for SA [3]. In addition, SA can be employed as a reagent in the synthesis of bio-polymers such as polybutylene succinate and polyurethane [4]. Although bio-based SA production offers environmental advantages, its successful commercialisation is dependent on cost-competitive production compared to the traditional petrochemical route. To this end, a conversion process is required where a microbial host efficiently converts renewable feedstock into SA.

Various microbial strains have been investigated for SA production with the most promising being *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens, Mannheimia succiniciproducens* and recombinant strains of *Escherichia coli* [5]. Despite competitive performance achieved by all these organisms, *A. succinogenes* has received substantial interest due to its ability to naturally produce SA at appreciable titres, yields and productivities. Furthermore, *A. succinogenes* is tolerant to high acid concentrations [6], is able to convert a variety of carbohydrates to SA [7], and consumes CO_2 in SA synthesis [8].

Given that CO_2 is a co-substrate in the production of succinic acid, the effect of CO_2 supply on SA production was studied in early publications on *A. succinogenes* [7,8]. Diffusion of dissolved CO_2 across the cell membrane is the main transport mechanism for inorganic carbon supply to the cell, since HCO_3^- permeation through the lipid membrane is insignificant [9,10]. Accordingly, the transient concentration of dissolved CO_2 in the broth (C_{CO_2}) is the main driver for CO_2 uptake by the cell. During fermentation, C_{CO_2} will decrease if the rate of CO_2 uptake by the cell exceeds the rate of CO_2 supply to the fermentation broth. CO_2 can either be supplied as a gas, requiring gas-liquid mass transfer, or in the form of a carbonate (i.e. HCO_3^-, CO_3^{2-}) in the liquid medium. Carbonate salts that are insoluble in water, such as $MgCO_3$ and $CaCO_3$, are often used as a source of CO_2 in fermentation media [8,10,11]. For these cases, the dissolution rate of the solid will be the rate determining step since the carbonate-bicarbonate-CO_2 equilibrium is rapidly established. Under typical fermentation conditions (pH = 6.8, T = 37 °C and CO_2 atmosphere at 1 bar) the dissolved carbonate concentration is low, while bicarbonate and dissolved CO_2 are present at approximately

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Nomenclature

C_{AA}	Acetic acid concentration g L^{-1}
$C^{eq}_{CO_2}$	CO_2 concentration at equilibrium mol L^{-1}
C_{CO_2}	Dissolved CO_2 concentration in broth mol L^{-1}
$C^{*}_{CO_2}$	Dissolved CO_2 concentration at saturation mol L^{-1}
$C^{P}_{CO_2}$	Dissolved CO_2 concentration at productivity threshold mmol L^{-1}
$C^{Y}_{CO_2}$	Dissolved CO_2 concentration at yield threshold mmol L^{-1}
$C^{feed}_{H^+}$	Proton concentration in feed mol L^{-1}
C_{H^+}	Proton concentration in broth mol L^{-1}
$C^{eq}_{H^+}$	Hydronium ion concentration at equilibrium mol L^{-1}
$C^{eq}_{H_2CO_3}$	H_2CO_3 concentration at equilibrium mol L^{-1}
$C^{eq}_{HCO_3^-}$	HCO_3^- concentration at equilibrium mol.L^{-1}
C_{OH}	Sodium hydroxide concentration mol L^{-1}
C_{SA}	Succinic acid concentration g L^{-1}
f_4	Fraction of total carbon flux in the C_4 pathway
f_{SA}	Fraction of phosphoenolpyruvic acid converted to succinic acid
H_0	Henry's constant in pure solvent kPa L mol^{-1}
K_1	Equilibrium constant – carbonic acid formation mol L^{-1}
K_2	Equilibrium constant – bicarbonate formation mol L^{-1}
K_3	Equilibrium constant – carbonate formation mol L^{-1}
K_4	Equilibrium constant mol L^{-1}
$k_g a_g$	Gas-based mass transfer coefficient h^{-1}
$P^{*}_{CO_2}$	CO_2 partial pressure kPa
Q	Overall volumetric flow rate mL min^{-1}
Q_D	Sodium hydroxide flow rate mL min^{-1}
Q_{feed}	Feed flow rate mL min^{-1}
q_{Glc}	Glucose consumption rate g L^{-1} h^{-1}
q_{SA}	Succinic acid productivity g L^{-1} h^{-1}
r_{CO_2}	Rate of CO_2 transfer mol L^{-1} h^{-1}
V	Reactor volume mL

equal concentrations. The highest possible C_{CO_2} value is determined by the CO_2 partial pressure in the gas phase and is in the vicinity of 20 mM for pure CO_2 at atmospheric pressure [10–12].

It has been shown that the supply of CO_2 influences SA productivity and catabolite distribution in *A. succinogenes* and *M. succiniciproducens* fermentations [8,10,11,13]. All these studies were performed in batch fermentations where the initial carbonate/bicarbonate concentrations and/or CO_2 partial pressures were varied. Although these studies demonstrate that carbonate supply can have a detrimental effect on the fermentation outcome, the mechanism of the effect is unclear. In the majority of the experiments performed in these studies, only the final cumulative outcome of the batch fermentation is reported. However, the rate of formation of succinic acid varies appreciably within a batch run as there is an initial growth period followed by product inhibition towards the latter stages of the fermentation [6,14]. Accordingly, the uptake rate of CO_2 remains variable throughout the fermentation likely causing fluctuations in C_{CO_2}. In one study [10], the CO_2 supply is shown to be stoichiometrically limited by the initial concentration of carbonates (since carbonates are not replenished during the fermentation), thereby causing a CO_2 shortage before the fermentation is complete. However, as mentioned above only the final, cumulative effect is reported. Variations in CO_2 partial pressure can be used to estimate the value of C_{CO_2} at saturation, although this does not guarantee that the broth is saturated because gas-liquid mass transfer can be rate controlling leading to C_{CO_2} falling below the saturation value.

Proper design and scale-up of a succinic acid fermentation process cannot rely solely on the results of lab-scale systems. In addition, it is important that the supply of CO_2 be linked to the transient value of C_{CO_2} which drives the inorganic carbon supply. When opting for a gaseous CO_2 supply, gas-liquid mass transfer should be carefully considered in order to achieve adequate C_{CO_2} values without excessive (and expensive) sparging schemes. For carbonate supply, the influence of mixing on the dissolution rate of the solids should be considered in order to maintain the required C_{CO_2} value in the medium. Both these routes require proper understanding and quantification of the effect of C_{CO_2} on succinic acid yield and cell-based productivity. To date, studies have not addressed these crucial relationships but only hint at the importance of CO_2 supply in SA fermentations.

In the current paper, the above mentioned shortcoming in *A. succinogenes* fermentation literature is addressed. Continuous fermentations were performed to enable steady-state analysis of the influence of C_{CO_2} on succinic acid fermentations with *A. succinogenes*. Steady-state conditions ensure that C_{CO_2} remains constant thereby allowing for a more accurate assessment of its influence on yield and cellular productivity. Gas-liquid mass transfer measurements were performed to calculate C_{CO_2} at various steady-state conditions. The amount of immobilised biomass was controlled by operating at glucose-limiting conditions whereby the biomass content formed over extended periods of operation was countered by the amount of biomass that washed out of the fermenter. Mass balances were performed to ensure that all the major metabolites were accounted for.

2. Materials and methods

2.1. Organism and fermentation medium

Cultures of *Actinobacillus succinogenes* 130Z (DSM 22257; ATCC 55618), acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ), were maintained in 66% v/v glycerol solutions at −40 °C. Inoculum was prepared in sterilised tryptone soy broth at 30 g L^{-1} and incubated at 37 °C and 150 rpm for 16 to 24 h in 30 mL sealed vials. Prior to inoculation, inoculum was analysed by HPLC to ensure culture purity and consistent metabolite distributions.

All chemicals were obtained from Merck KgaA unless indicated otherwise. The fermentation medium consisted of three parts: (1) a nutrient and salts mixture, (2) a carbohydrate solution and (3) a phosphate buffer. The nutrient and salts mixture was based on [15] and consisted of (in g L^{-1}): 16.0 yeast extract, 1.0 NaCl, 0.2 $MgCl_2{\cdot}6H_2O$, 0.2 $CaCl_2{\cdot}2H_2O$, 1.36 sodium acetate, 0.16 $Na_2S{\cdot}9H_2O$ and 0.5 – 1 mL L^{-1} Antifoam Y-30 (Sigma-Aldrich, Germany). The phosphate buffer comprised 1.6 g L^{-1} KH_2PO_4 and 0.8 g L^{-1} K_2HPO_4. Glucose solutions were prepared at 25 g L^{-1}.

2.2. Continuous fermentations

Three fermentations were performed in a custom, externally-recycled bioreactor, similar to that used in [16]. The volume was maintained at 358 mL by means of an overflow tube connected to an exit pump. pH was measured by a Ceragel CPS71D glass electrode (Endress + Hauser, Germany) connected to a Liquiline CM442 unit (Endress + Hauser, Germany) and controlled at 6.80 ± 0.01 by the addition of 10 N NaOH. Temperature was measured by the pH