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Substrate and product inhibition kinetics in succinic acid production by *Actinobacillus succinogenes*

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ABSTRACT

The inhibition of substrate and products on the growth of *Actinobacillus succinogenes* in fermentation using glucose as the major carbon source was studied. *A. succinogenes* tolerated up to 143 g/L glucose and cell growth was completely inhibited with glucose concentration over 158 g/L. Significant decrease in succinic acid yield and prolonged lag phase were observed with glucose concentration above 100 g/L. Among the end-products investigated, formate was found to have the most inhibitory effect on succinic acid fermentation. The critical concentrations of acetate, ethanol, formate, pyruvate and succinate were 46, 42, 16, 74, 104 g/L, respectively. A growth kinetic model considering both substrate and product inhibition is proposed, which adequately simulates batch fermentation kinetics using both semi-defined and wheat-derived media. The model accurately describes the inhibitory kinetics caused by both externally added chemicals and the same chemicals produced during fermentation. This paper provides key insights into the improvement of succinic acid production and the modelling of inhibition kinetics.

1. Introduction

In recent years, fermentative production of succinic acid has received increasing interest because of its potential as a platform chemical for the production of various value-added derivatives. Succinic acid and its derivatives have numerous applications, including surfactants, detergents, electroplating, food, pharmaceutical, antibiotics, amino acids and vitamins [1]. It can be produced in microbial fermentations by a number of microorganisms, such as *Actinobacillus succinogenes* [1,2], *Anaerobiospirillum succiniciproducens* [3,4], *Mannheimia succiniciproducens* [5] and recombinant *Escherichia coli* [6]. Of all these, *A. succinogenes* is one of the most promising succinic acid producers due to its distinctive ability to produce succinic acid naturally from a broad range of carbon sources [1,7]. It produces succinic acid as the major fermentation product, along with acetic acid, pyruvic acid, formic acid and ethanol as minor products [1]. Many studies have focused on strain improvements and manipulation of medium and feeding control to improve succinic acid production [2,8–11]. Few have addressed the influence of substrate and/or product inhibition in the succinic acid fermentation. Very recently, however, Song et al. [12] proposed a kinetic model for batch fermentations using another succinic acid producer, *M. succiniciproducens* MBEL55E. In this model, a modified Monod equation that incorporates both substrate and product inhibition was used to describe the cell growth. The Luedeking–Piret model was used to simulate the formation of fermentation products, such as acetic, formic, lactic and succinic acids.

Urbance et al. [10] reported that *A. succinogenes* could tolerate up to 160 g/L initial glucose concentration in batch fermentation. Similarly, it would grow in medium consisting of 100 g/L sugar mixture from pretreated cane molasses that contained fructose, glucose and sucrose [11]. Significant decreases in biomass, succinic acid production and sugar utilisation were observed when the initial sugar concentration was over 65 g/L. Furthermore, *A. succinogenes* could tolerate up to 96 g/L disodium succinate hexahydrate, which is equivalent to 42 g/L succinic acid [1,13]. Fermentations in a 'plastic composite support biofilm bioreactor' also confirmed that succinic acid production was halted when about 40 g/L succinic acid was produced [10]. In our previous investigation, significant inhibition of cell growth was observed when succinic acid concentration was as low as 20 g/L, most likely due to the inhibitive effect of other fermentation end-products [14]. A systematic investigation of the substrate and product inhibition is, therefore, crucial for the development of an effective control strategy to improve succinic acid production and the modelling of inhibition kinetics.

In this study, the inhibition potentials of substrate and products on *A. succinogenes* in succinic acid fermentation are quantitatively examined. A kinetic model is proposed to describe these effects on

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Nomenclature

a_i	exponent of inhibitory product
C_{Pi}	concentration of inhibitory product (g/L)
C^*_{Pi}	critical concentration of inhibitory product above which cells do not grow (g/L)
C_S	initial substrate (glucose) concentration (g/L)
$C_{S,max}$	highest experimentally observed initial substrate concentration at which growth still occurs (g/L)
$C_{S,min}$	minimum substrate concentration in which lag time arise (g/L)
C^*_S	critical glucose concentration above which cells do not grow (g/L)
DCW	dry cell weight (g/L)
FAN	free amino nitrogen (g/L)
K_S	Monod or substrate saturation constant (g/L)
m	power constant
m_e	maintenance coefficient (g substrate/g biomass h)
n	power constant
OD_{660}	optical density at 660 nm
S	glucose concentration (g/L)
t	time (h)
T_l	lag time (h)
X	biomass concentration (g/L)

Greek letters

α_{Pi}	constant for growth associated term of product formation (g product (g biomass)$^{-1}$)
β_{Pi}	constant for non-growth associated term of product formation (g product (g biomass h)$^{-1}$)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
η, κ, λ	empirical constants of Eq. (5)
δ	constant of Eq. (8) (g substrate (g biomass)$^{-1}$)
γ	constant of Eq. (8) (g substrate (g biomass h)$^{-1}$)

cell growth. The critical concentrations of glucose as substrate and acetate, ethanol, formate, pyruvate and succinate as end-products were determined. Furthermore, the model is verified with data from batch fermentations using both semi-defined and wheat-derived media.

2. Materials and methods

2.1. Microorganism and growth conditions

All chemicals used throughout this study were obtained from Sigma, UK and Fisher Scientific, UK, except where otherwise specified. *A. succinogenes* (ATCC 55618) was obtained from the American Type Culture Collection (ATCC, Manassasa, VA, USA). Inoculum was prepared by incubating *A. succinogenes* cells from cryopreservation vial in 100 mL Duran bottles containing 50 mL of trypticase soya broth (TSB, Fluka, BioChemika, Buchs, Switzerland) at 30 °C (recommended ATCC cultivation procedure) on a rotary shaker of 100 rpm for 48 h.

2.2. Media and conditions

Experiments for determining the inhibitory effects of substrate and products were carried out in small anaerobic reactors (SARs), each containing 45 mL semi-defined medium. The semi-defined medium contained (L^{-1}) [15]: glucose, 30 g; yeast extract (Fisher BioReagents, Fisher Scientific, Loughborough, UK), 5 g; $NaH_2PO_4{\cdot}H_2O$, 1.16 g; Na_2HPO_4, 0.31 g; NaCl, 1.0 g; $MgCl_2{\cdot}6H_2O$, 0.2 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; B_{12}, 1 μg; biotin, 20 μg; folic acid, 20 μg; thiamine, 50 μg; riboflavin, 50 μg; niacin, 50 μg; pantothenate, 50 μg; *p*-aminobenzoate, 50 μg; lipoic acid, 50 μg; B_6, 100 μg, $MgCO_3$, 30 g, silicone antifoam, 1 mL. A series of experiments was conducted using a modified semi-defined medium with addition of substrate (glucose) or end-products (ethanol, sodium acetate, sodium formate, disodium succinate hexahydrate and sodium pyruvate) in various concentrations as listed in Table 1. Fermentation samples were taken every 2–3 h to measure optical density and glucose concentration. Fermentations ended when either glucose was completely depleted or no change in glucose concentration in 12 h. The pH of the medium was 7.2 after autoclaving for 20 min at 121 °C. Separately autoclaved glucose solution was added aseptically to the medium to make up the desired glucose concentration ranging from 0 to 160 g/L. The SARs were placed on a rotary shaker at 100 rpm and incubated at 37 °C. The fermentation broth was sparged with 0.2 vvm CO_2 and the inoculum size was 2% (v/v). The fermentations in SARs were carried out in triplicate.

Two batches of bacterial fermentations were carried out at 37 °C with a working volume of 0.5 L semi-defined and wheat-derived media separately in a 1.8-L bench-top bioreactor (Electrolab 351, Tewkesbury, UK). The composition of semi-defined medium was identical to the one used in the SARs, except for a glucose concentration of around 85 and 10 g/L yeast extract. The wheat-derived medium used in this study was generated from a soft wheat variety (*Consort*), harvested in 2003 and supplied by Fisher Seed and Grain Limited (Cranswick, East Yorkshire, UK). The composition of wheat-derived medium composed of 300 mL flour hydrolysate containing around 170 g/L glucose and 200 mL fungal autolysate containing 1.6 g/L free amino nitrogen (FAN). The latter corresponds to the FAN content of a 32 g/L yeast extract solution. Prior to autoclaving, 30 g/L $MgCO_3$ was also added to the medium as a neutral pH buffer for the fermentation. The detailed procedure for preparing the wheat-derived medium has been described in an earlier publication [14]. The pH was automatically controlled at 6.6–6.8 with the addition of 10 M NaOH solution. The broth was sparged with 0.5 vvm CO_2 and agitated at 200 rpm. The inoculum size for the batch fermentation was 4% (v/v).

Table 1
Summary of experimental conditions

Type of investigation	Media	Scale of bioreactors	Initial glucose (g/L)	Externally added chemicals[a] (g/L)
Substrate inhibition	Semi-defined	Small anaerobic reactors	0–160	–
Products inhibition	Semi-defined	Small anaerobic reactors	30	Disodium succinate hexahydrate: 0–100; ethanol: 0–40; sodium acetate: 0–48; sodium formate: 0–15; sodium pyruvate: 0–72
Combined substrate and products inhibition	Semi-defined	Bench-top bioreactor	85	–
	Flour hydrolysate and fungal autolysate	Bench-top bioreactor	100	–

[a] The fermentation medium was modified by addition of end-products separately.