Experimental and Modelling Studies of the Bioconversion of Glycerol to Succinic Acid by Actinobacillus Succinogenes

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Abstract

An unstructured kinetic model for the growth of *Actinobacillus succinogenes* on glycerol as the only carbon source is proposed in this study. The model describes cell growth considering both substrate and product inhibition. and its parameters are estimated by fitting the model predictions to experimental data. Substrate consumption and product formation rates are described by the Luedeking-Piret model. The main product of the process is succinic acid while by-products like acetate, formate and ethanol have very low concentrations. The main environmental factors that affect the bioprocess were examined and optimum conditions in terms of yield, final succinic acid concentration and productivity were evaluated by a factorial experimental procedure. The examined parameters were the supply of CO_2, the pH level and the redox balance. Experiments using different initial glycerol concentrations at the "optimum" environmental conditions were carried out and simulation studies were performed using the proposed model.

1) Introduction

Biodiesel is an alternative transport fuel which is made from renewable sources such as vegetable oils and animal fats. Recently, biodiesel production has received increasing attention due to national and international legislations, increasing petroleum prices, depletion of conventional fuels, concerns about food-to-fuels production and potential environmental benefits of biofuels. As the demand and the production of biodiesel grow fast, the development of methods to increase the sustainability of the biodiesel industry becomes an urgent topic.

Glycerol which is the main by-product of the biodiesel industry represents 10% (w/w) of the biodiesel produced and constitutes the basic bottleneck because of its currently limited applications due to the impurities that it contains [1]. In the last two decades, 1.3-propanediol has received increasing interest as a potential bulk chemical produced from crude glycerol since this bioconversion can achieve high final product concentrations and high productivities [2], [3]. The large potential that bio-processing exhibits, has instigated research efforts into further prospects of crude glycerol. Recently, fermentation studies of glycerol have examined the production of other products such as ethanol [4] and succinic acid.

Succinic acid is a C4 linear saturated dicarboxylic acid and is currently produced by petrochemical precursors through reduction of maleic anhydride and by microbial fermentation from glucose and/or other sugars [5]. Succinic acid has numerous applications and it can be used as surfactant, detergent, ion chelator and also in the food and pharmaceutical market. An extra advantage of the succinate bioprocess is that the microbial formation of succinic acid requires the fixation of a greenhouse gas: carbon dioxide [5].

Fermentation processes are affected by many environmental factors and usually produce a number of by-products. Research for the optimal environmental conditions in bio-

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processes not only targets the maximisation of the desired product but also the minimisation of side products. From the environmental and physiological conditions that seem to affect the succinic acid production the most important are the amount of dissolved CO_2 in the fermented bed, the availability of electron donors, the pH values during the fermentation and the initial substrate concentration [6].

In particular, M. J. Van der Wert et al [6] have examined the effect of carbon dioxide on the succinic acid production on *Actinobacilus succinogenes* 130Z strain by supplying initially amounts of magnesium carbonate in the bioreactor. Results showed that there is a direct relationship between CO_2 and succinic acid production and simultaneously an inverse behaviour on the side-products formation (acetic acid, formic acid and ethanol). Furthermore, J B McKinlay et al [7] suggested the provision of hydrogen during the fermentation as electron donor to achieve a certain redox balance. According to the overall succinate reaction, four electrons [H^+] are required to form succinic acid from glucose, thus electron supply in the form of hydrogen as gas during the fermentation resulted in a significantly higher succinic acid production.

Samuelov et al [8] studied the influence of pH and CO_2-HCO_3 levels on succinic acid production by using the bacterium *Anaerobiospirillum succiniciproducens*. They discovered that from neutral towards acidic pH (7.2-6.2) and from low to high amounts of CO_2 the succinic acid production was increased in favour of lactic acid and ethanol. The pH, during the succinate bioprocess, decreases due to the production of acids and also affects the solubility of CO_2 and H_2 in the medium.

The succinic acid production has been investigated by a set of bacteria such as genetically modified *E.coli*, *Anaerobiospirillum succiniciproducens*, *Mannhemia succiniciproducens* and *Actinobacillus succinogenes*. Among them, *Actinobacillus succinogenes*, a bacterium derived from the bovine rumen, is considered to be a natural succinic acid producer which can produce and accumulate higher amounts of the desired product from glucose as well as from a wide range of sugar-substrates including lactose, xylose, arabinose, fructose and sorbitol [10]. *Actinobacillus succinogenes* is recognised as one of the most suitable microorganisms for the industrial production of succinic acid due to its high tolerance for elevated product concentrations [10].

Until now, there have been very few works studying the dissimilation of glycerol for the succinate production [9]. Most of them have utilised low initial glycerol concentrations due to the decrease of the uptake rate at high glycerol concentrations. The aim of this study is to present an unstructured model that can predict the microbial growth, the substrate and products concentrations during a series of batch fermentations of *Actinobacillus succinogenes* with several initial glycerol concentrations. A factorial design was implemented to determine the optimum environmental conditions of the bioprocess in terms of yield, final succinate concentration and productivity. The necessary kinetic parameters for the model are estimated by fitting the model predictions to experimental observations.

2) Materials and Methods

2.1) Culture strain and inoculum preparation

Actinobacillus succinogenes (ATCC 55617) was obtained from the American type culture collection and it was preserved in cryopreservation vials in -70^{o}C. Preculture of the strain was performed in 100ml Duran bottles containing 50 ml of trypticasein soy broth at 30^{o}C on a rotary shaker at 100 rpm for 1-2 days. Cultivation of the strain was continued using small anaerobic reactors (SAR), each containing 48ml of semi-defined medium [10].

Glycerol was used instead of glucose, as the carbon source in a medium containing per litre: glycerol 10g, yeast extract 5g, $NaH_2PO_4 \cdot H_2O$, 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; B12, 1_g; biotin, 20_g; folic acid, 20_g; thiamine, 50_g; riboflavin, 50_g; niacin, 50_g; pantothenate, 50_g; *p*-aminobenzoate, 50_g; lipoic acid, 50_g; B6, 100_g, $MgCO_3$, 10 g, silicone antifoam, 1mL. Glycerol solution and semi defined medium were separately autoclaved for 15 minutes at 121^oC. After inoculation from TSB bottles the SARs were placed on a rotary shaker at 100 rpm and incubated at 37^oC for 1-2 days. The volume of the inoculum was around 4 %v/v and CO_2 gas was supplied during the fermentation.

2.2) Batch fermentations in Bench-Top Bioreactor

Batch fermentations were carried out in a 1.8 litres bench-top bioreactor containing a working volume of 0.5 litres. The composition of the medium was similar to the one used in the SARs except for small changes due to the specific experimental design. Thus, glycerol, MgCO3 and $NaBH_4$ concentrations were ranged from (0-40 g/l), (10-30 g/l) and (0-20 g/l) while yeast extract concentration was set to 10 g/l. Moreover, the pH was automatically controlled at a range 6.2-7.0 with the addition of HCl and NaOH (10M solutions). Gaseous CO_2 was supplied at a flow rate of 0.4vvm. The agitation speed set to 200rpm. The inoculum size for the batch fermentation was 10% and all batches were duplicated.

2.3) Analytical techniques

Cell growth was determined spectrophotometrically (spectrophotometer UVmini 1240, Shimadzu,Europa, Germany) by measuring the optical density (OD) at a wave length of 660. In order to ensure that none of the $MgCO_3$ remained undissolved in the sample, 0.5mL of the fermentation sample was diluted with 2 mL of 7% (v/v) hydrochloric acid (HCl) to form soluble magnesium chloride and carbon dioxide. The relationship between 1 OD_{660} and dry cell weight (DCW) per litre was found to be 0.626 g-DCW/litre.

Glycerol concentration was analysed by using a GL6 Analyser (Analox Instruments, UK) which measures the enzymatic oxygen consumption rate. Fermentation products such as succinic acid, acetic acid , formic acid , pyruvic acid and propionic acid were measured by High Perfonmance Liquid Chromatographer (Star Varian Chromatography Workstation) with a UV detector (Prostar 330 PDA). A Hi-Plex H 8 μm 300 × 7.7 mm (Polymer Laboratories) column was used to separate the components preceded by a guard column (PL Hi-Plex H Guard column 50 × 7.7 mm, Polymer Laboratories) at a flow rate of 0.6ml/min using a mobile phase of 0.1% v/v trifluoroacetic acid (TFA) at a column temperature 60^oC.

2.4) Factorial design description

The aim of the factorial design is to determine the optimal environmental conditions of the bioprocess in terms of product yield, final concentration and productivity. Moreover, it indicates the crucial environmental parameters that affect the bioprocess and the interactions between them [11] .To do so, a 2^3 factorial design was suggested leading to 8 experiments. Four extra experiments were also carried out at the centre point. These parameters are selected to be the CO_2 supply, the pH level and the redox balance. The values and range of these controlling parameters were selected according to preliminary experiments and previous studies and are shown in Table 1.

Table1: Levels and range of the factorial design variables

Variables	Range and Levels		
	-1	0	+1

$MgCO_3$ (g/l)	X_1	10	20	30
pH	X_2	6.3	6.6	6.9
$NaBH_4$ (g/l)	X_3	0	10	20

The controlling parameters were coded according to the following equation:

$$X_i = \frac{X_i^r - X_i^{ro}}{\Delta X} \quad (1)$$

where X_i is the coded value of the variables, X_i^r and X_i^{ro} are the real values of the variables in level -1, +1 and 0 respectively and ΔX is the step change.

The following linear polynomial was used for the estimation of the dependent variable.

$$Y_i = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta ij X_i X_j + \beta_{ijk} X_i X_j X_k \quad (2)$$

where Y_i is the estimated variable, β_o is the intercept of the polynomial, β_i is the linear coefficients, and β_{ij} and β_{ijk} are the interaction coefficients.

The adequacy of eq.(2) was checked by the Fisher criterion with level of confidence 5% while the importance of the coefficients was checked by the student-t distribution also with a 5% level of confidence[2].

2.5) Model studies

A modified Monod model was used to describe the growth kinetics considering both substrate and product inhibition.

In preliminary experiments, it was estimated that there is an excessive substrate inhibition on microbial growth. Therefore, the following modified Monod model was used to describe the substrate inhibition [12],[13],[14]:

$$\mu = \mu_{\max} \cdot \frac{S}{S + K_S + (S^2 / K_I)} \quad (3)$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (g/L), K_s is the substrate saturation constant (g/L) and K_I is the substrate inhibition constant (g/L).

Furthermore, during the fermentation the formation of products can act as an inhibition factor and can gradually affect the cell growth. Thus, in addition to eq.(1) a product inhibition term was introduced [13].

$$\mu = \mu_{\max} \cdot (1 - \frac{P_i}{P_i^*})^n \quad (4)$$

where P is the concentration of the product during the fermentation (g/L), P^* is the succinic acid concentration above which cells do not grow (g/L), *n* is the empirical constant which describes the linearity or not of the system (dimensionless) and the index indicates the number of by-products.

Combining eq.(3) and eq.(4), a final extended expression is developed (eq. 5) which takes into consideration substrate inhibition and multi inhibition effects of products.

$$\mu = \mu_{\max} \cdot (\frac{S}{S + K_S + (S^2 / K_I)})(1 - \frac{P_i}{P_i^*})^n \quad (5)$$

The cell growth rate (dX/dt) can then be described by equation (6).

$$\frac{dX}{dt} = \mu \cdot X \quad (6)$$

where X is the cell concentration (g/l)

Finally, the substrate consumption rate (dS/dt) and the products formation rate (dP/dt) can be described by Luedking-Piret model (eq. 7 and 8 respectively).

$$\frac{dS}{dt} = -\alpha \frac{dX}{dt} - \beta X \quad (7)$$

$$\frac{dP}{dt} = \gamma \frac{dX}{dt} + \delta X \quad (8)$$

where α is the constant term for substrate consumption (g-S/g-X), β is the maintenance coefficient of the substrate (g-S/g-X h), γ is the constant growth term of product formation (g-P/g-X) and δ is the maintenance coefficient of the products (g-P/g-P h).

2.6) Parameter Estimation

The eight parameters of the model proposed were estimated using nonlinear, weighted, least squares method by minimizing the sum of squared errors between the estimated and experimental values. Simulated annealing (SA), a stochastic optimization algorithm implemented in MATLAB was used. Simulated annealing can avoid local minima and probabilistically converges to the region around the global minimum. A deterministic optimisation step using Successive Quadratic Programming was implemented next to pinpoint precisely the global minimum. In order to avoid potential unrealistic optima all parameters were constrained within limits found in the literature for similar systems [2], [12], [13], [14]. These bounds are presented together with the values obtained from the combined optimization algorithm in Table 2. The initial values of the ODEs were calculated according to the initial conditions of each experiment.

3) Results and Discussion

Preliminary experiments on glycerol for succinic acid production by *Actinobacillus succinogenes* showed that this substrate, compared to glucose, is not very amenable to biodegradation and results in low productivities and final succinic acid concentrations due to possible substrate inhibition. In this study we have developed a priori a factorial design experiment to examine the effect of the most crucial parameters.

Factorial design results indicated the significance of the most important parameters (CO_2 supply, pH value and redox balance) and their interactions. Succinic acid was affected by all three parameters and its production was amended due to $MgCO_3$ and $NaBH_4$ addition at low pH values. According to Student's t-test $MgCO_3$ had the strongest positive linear effect on the predicted values followed by the pH values and $NaBH_4$. Linear and interaction coefficients were calculated from the obtained experimental values and optimum conditions were estimated by optimising the predicted value (eq 2) in terms of productivity, final succinic acid concentration and yield. Optimum conditions were assessed to be at 40 g/L $MgCO_3$, 25 g/L $NaBH_4$ and 6.4 pH value. The experiments that were carried out at this point to validate the results illustrated good agreement with the predicted values (data not shown). Furthermore, the interactions between the controlling parameters were estimated. It seems that there is a strong interaction between both $NaBH_4$ and $MgCO_3$ and the pH value. Significant interactions were also noted between $NaBH_4$ and $MgCO_3$.

At the optimum conditions, batch fermentations with initial glycerol concentration of 0, 5, 10, 15, 20, 30, 40, 50 g/L were carried out and the unstructured model described above was used to simulate the experimental data. The proposed model includes both substrate and product inhibition. Simulation results using model for cell growth, substrate concentration and succinic acid formation are shown in figure 1. Since in almost all the experiments the by-product formation was very low compared with succinic acid; acetic acid, formic acid, ethanol and pyruvic acid product inhibitions were not included in the model.

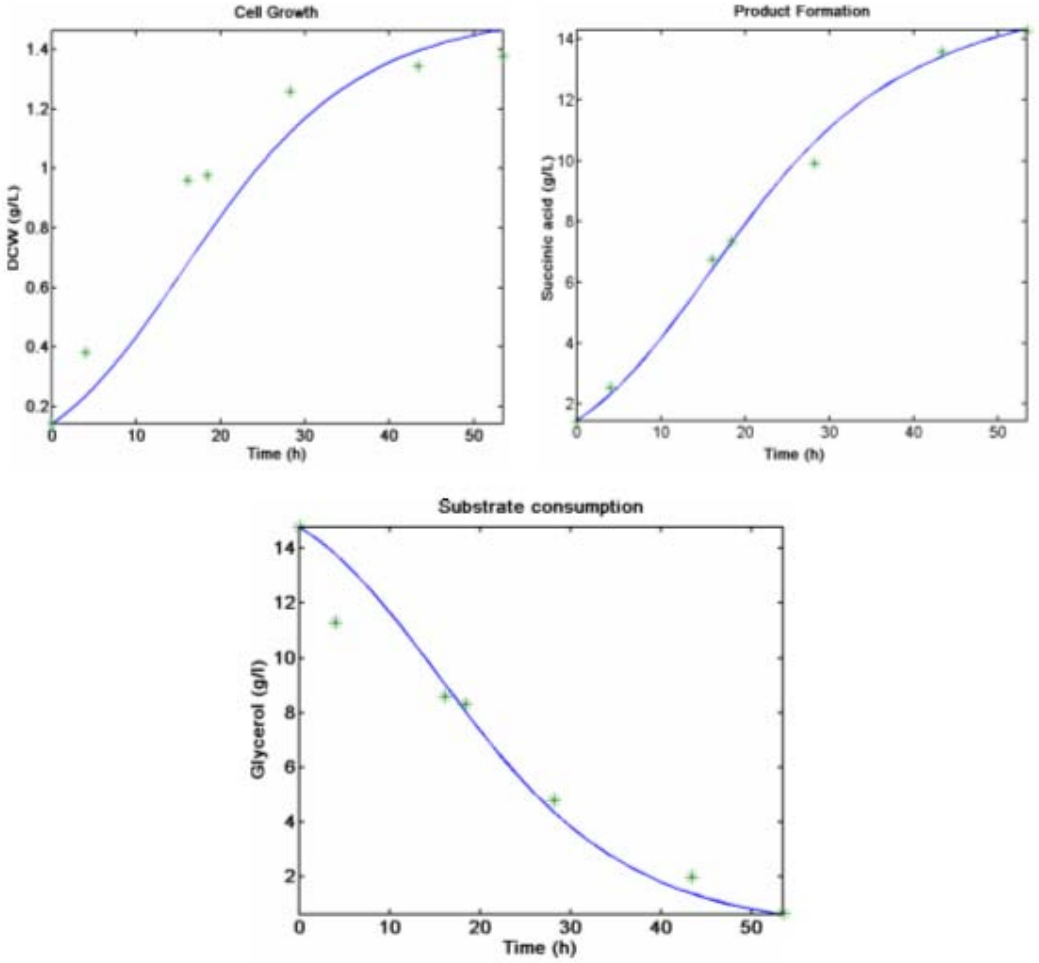

Figure 1: Experimental and simulation data of glycerol, biomass and succinic acid

The critical concentration of succinic acid P* above which cells do not grow was measured in a previous study [12] and it was found to be equal to 155 g/L; this value indicates the major tolerance of *Actinobacillus succinogenes* to high succinic acid concentrations. The parameters of the model were estimated using a combination of stochastic and deterministic optimisation and the obtained results are shown in table 2. The model can adequately predict the kinetics of the fermentation for a wide range of initial glycerol concentrations. Although, it is the first time a model has been developed for this system, the obtained parameter values were compared with results from similar studies such as 1,3-PD production [3], and succinic acid production from glucose [12] , [13] and showed good agreement. Nevertheless, it should be noted that parameter values of such an unstructured model have limited physical significance due to the fact that they are based

on an overall reaction for all the phenomena involved in the conversion of substrate to biomass.

Table 2: Parameters of the model

Parameters	Units	Values	Description	Bounds
μ_{max}	h^{-1}	1.39	Maximum specific growth rate	0.07-1.6
K_s	g/L	2.5	Substrate saturation constant	0.04-4
K_I	g/L	80	Substrate inhibition constant	1-80
n	-	0.5	empirical constant	0.4-1.4
α	g-S/g-X	4.67	Constant term for substrate	0.6-5.4
β	g-S/g-X h	0.001	Substrate maintenance coefficient	0.001-1.2
γ	g-P/g-X	2.02	Constant growth term of product	0.4-2.8
δ	g-P/g-X h	0.018	Product maintenance coefficient	0.001-1.2

The maximum product yield, final concentration and productivity were found to be 0.75mol-S.A/mol-Gly, 28 g/L S.A. and 0.4 g-S.A./L h respectively on a medium containing initially 29.3 g/L glycerol. No cell growth was observed above 40g/l initial glycerol concentration. However, these results are significantly lower than the corresponding ones for 1,3-PD production from glycerol [3] and for succinic acid production from glucose [12]. They are, however, the highest observed for this system and further experimental investigations to obtain better results are needed.

Conclusions

In this study, an empirical model was developed to describe *Actinobacillus succinogenes* growth on glycerol for succinic acid production. The modified Monod expression model includes substrate and product inhibition and can adequately predict batch fermentation production rates for a wide range of initial glycerol concentrations. Experiments were carried out at optimum environmental conditions indicated by a 2^3 factorial design. The parameter values obtained were in good agreement with values in similar studies. Although, succinic acid production from glycerol is less favourable compared to the formation of 1,3-propanediol from glycerine and succinic acid production from glucose, this process seems to be a promising alternative increasing the sustainability of biodiesel production. Further investigation is needed to optimise the bioprocess. Fed-batch fermentations and recycling biomass techniques may improve the process significantly. Also, more detailed models that can give insights into the intracellular reactions and phenomena are needed. These models can be used for optimising the succinic acid metabolic pathways simultaneously minimising by-product streams.

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