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Utilisation of glycerol to platform chemicals within the biorefinery concept: A case for succinate production

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In this study, a biorefinery concept is introduced for the production of platform chemicals by utilising the by-products of the biodiesel industry. An unstructured kinetic model for the bacterial growth of *Actinobacillus succinogenes*, which is our chosen biocatalyst, is proposed. The model describes cell growth and considers both substrate and product inhibition. The main product chosen here is succinic acid and by-products like acetate, formate and ethanol have insignificant low concentrations. Experiments on different initial glycerol concentrations at the same environmental conditions are carried out and simulation studies are conducted using the proposed model. Parametric values are estimated based on experimental results. Prior to that, the main environmental factors that affect the bioprocess are examined and beneficial conditions in terms of yield, final succinic acid concentration and productivity are assessed by a factorial experimental procedure.

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 increase in petroleum prices, supply issues for conventional fuels, potential environmental benefits of biofuels and national and international legislations. Thus, 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.
The main by-product of this process is glycerol which represents 10% of the biodiesel produced and constitutes the basic bottleneck as this crude product has limited applications due to the impurities that it contains (Thompson and He, 2006). During the last 20 years, research has been focused on the bioconversion of glycerol to 1,3-propanediol (Zeng et al, 2008), (Zheng et al, 1994). The result of this long-period research has finally led to the industrialization of the product and illustrated the potential of bio-processing. Therefore, researchers have started to examine alternative bio-products from glycerol such as succinic acid and ethanol.
Fermentation processes are affected by many environmental factors and usually produce many by-products. From the environmental and physiological conditions that seem to

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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 (Van der Werf et al, 1997).
The aim of this study is to present a model that can predict the microbial growth, and the substrate and products concentrations during a series of batch fermentations of *Actinobacillus succinogenes* with several initial glycerol concentrations. Prior to this, a factorial experimental procedure was implemented to determine the optimum environmental conditions in terms of yield, succinate final concentration and productivity.

2. Materials and methods

2.1 Inoculum preparation and cultivation conditions

Actinobacillus succinogenes (ATCC 55617) was obtained from the American type culture collection and it was preserved in cryopreservation vials in -70°C. Preculture of the strain was performed in 100ml Duran bottles containing 50 ml of trypticase in soy broth (TSB) at 30°C on a rotary shaker at 100 rpm for 1-2 days. Cultivation of the strain continued in small anaerobic reactors (SARs) each containing 45ml of chemically defined medium (Guettler et al, 1999). Glycerol was used as the carbon source and the medium contained 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, 1μg; biotin, 20μg; folic acid, 20μg; thiamine, 50μg; riboflavin, 50μg; niacin, 50μg; pantothenate, 50μg; *p*-aminobenzoate, 50μg; lipoic acid, 50μg; B6, 100μg, $MgCO_3$, 10 g, silicone antifoam, 1mL. The glycerol diluted in water and the rest of the medium were separately autoclaved for 15 minutes at 121°C. The SARs with the mixed medium were placed on a rotary shaker at 100 rpm and incubated at 37°C for 1-2 days. The inoculum was around 10 %v/v and CO_2 gas was supplied during the fermentation.

2.2 Bench-Top Bioreactor

Batch fermentations were also carried out in a 1.8 l bench-top bioreactor containing a working volume of 0.5 l. The composition of the medium was similar to the one used in the SARs except for small changes according to the experimental design. Here, yeast extract concentration was set to 10 g/l. Moreover, the pH was automatically controlled in a range 6.2-7.0 (according to the factorial design) with the addition of HCl 5M and NaOH 10M solutions. Gas CO_2 was supplied at a flow rate of 0.4vvm and agitation speed set at 200 rpm. The inoculum for the batch fermentation was 10% v/v and batches were carried out in duplicate.

2.3 Analytical methods

Cell growth was determined spectrophotometrically (spectrophotometer UVmini 1240, Shimadzu, Europa, Germany) by measuring the optical density (OD) at a wave length of 660. The linear relationship between OD_{660} and dry cell weight (DCW) per litre was found to be 0.626 g-DCW for OD_{660} equal to 1.
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 a High Performance Liquid Chromatographer (Star Varian Chromatography Workstation) with a UV detector (Prostar 330 PDA) and a Hi-Plex H 8 μm 300 × 7.7 mm (Polymer Laboratories) column.

2.4 Factorial design

The scope of the factorial design is to determine the optimal environmental conditions of the bioprocess in terms of product yield, final concentration and productivity. Furthermore, factorial design indicates the crucial environmental parameters that affect the bioprocess and the interactions between them (Cochran, W.C. and Cox, 1957). A 2^3 factorial design is suggested as an initial effort leading to 8 experiments. Four extra experiments were also carried out at the centre point (level 0, see Table 1). 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.

Table 1: Levels and range of the factorial design variables

Variables		Range and Levels		
		-1	0	+1
$MgCO_3$ (g/l)	X_1	5	10	15
pH	X_2	6.3	6.6	6.9
$NaBH_4$ (g/l)	X_3	0	5	10

The controlling parameters, X_i were normalised according to the following eq.(1):

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

where, X_i^r and are the values of the variables at level -1, +1, X_i^r the values at level 0 and $\Delta X = | X_i^r - X_i^r |$.

Moreover, the following polynomial was used for the estimation of the dependent variables.

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

where: Y_i are the estimated variables (yield, final succinic acid concentration, productivity), β_o is the intercept of the polynomial, β_i are the linear coefficients of the three parameters and β_{ij} are the interaction coefficients.

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

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 due to low substrate consumption rates. In addition, a product inhibition term was also introduced (Song et al, 2008) leading to the final extended model expression:

$$\mu = \mu_{\max} \cdot \left(\frac{S}{S + K_S + (S^2/K_I)}\right)\left(1 - \frac{P_i}{P_i^*}\right)^n \quad (3)$$

where S and P are the substrate and product concentration respectively (g/l) , P^* is the (by)product concentration above which cells do not grow (g/l), and the index indicates the number of by-products. (All other parameters are described in table 2). The cell growth rate (dX/dt) can simply be described by eq.(4).

$$\frac{dX}{dt} = \mu \cdot X \quad (4)$$

Finally, the product formation rate (dP/dt) can be described by the Luedking-Piret model and the substrate consumption rate (dS/dt) can be by an overall carbon-mass balance (eq. (5) and (6) respectively). (All parameters are given in table 2).

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (5)$$

$$\frac{dS}{dt} = -\frac{1}{Y_X}\frac{dX}{dt} - m_s X - \frac{1}{Y_{SA}}\frac{dP}{dt} \quad (6)$$

2.6 Parameter Estimation

The nine optimisation parameters of the model proposed were estimated by minimizing the sum of squared errors between the estimated and experimental values. The corresponding optimisation problem was solved using first simulated annealing, a stochastic optimization algorithm implemented in Matlab, which can avoid local minima and probabilistically compute the region of the global minimum. Deterministic optimisation was subsequently implemented, using the Successive Quadratic Programming method to compute precisely the global minimum and the final parameter values. In order to avoid potential unrealistic optima all parameters were constrained within limits found in the literature for similar systems (Song et al 2008), (Lin et al 2008) (table 2). The initial values of the ODEs (eq. 4-6) were calculated according to the initial conditions of each experiment.

3. Results and Discussion

The results of the factorial design indicated the significance of the most important parameters (CO_2 supply, pH value and redox balance) and their interactions. Succinic acid production was affected by all three parameters and its production was enhanced at high $MgCO_3$ and $NaBH_4$ levels 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 values of eq.(2) in terms of productivity, final succinic acid concentration and yield. The best conditions are currently assessed to be at 18 g/L $MgCO_3$, 8 g/L $NaBH_4$ and 6.4 pH value. At these environmental conditions, batch fermentations were performed in both SARs bench-top reactors with initial glycerol concentrations of 0, 5, 10, 15, 20, 30, 40, 50 g/l. Simulations of the proposed model for cell growth, substrate concentration and succinic acid formation for SARs and Bench-top Reactors 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.

The critical concentration of succinic acid P* above which cells do not grow was measured in a previous study (Lin et al, 2008) and its equal to 155 g/L; this value indicates the high tolerance of *Actinobacillus succinogenes* to high succinic acid concentrations.

The parameters of the model were estimated using both stochastic and deterministic optimisation methods as described above and the obtained results are shown in table 2. The model can adequately predict the fermentation kinetics in a wide range of initial glycerol concentrations. Although it is the first time that modelling studies were developed on such a system, the obtained parameter values were comparable with results obtained in similar studies that used glucose as a substrate (e.g. Lin et al., 2008).

The maximum product yield, final concentration and productivity that have been obtained so far were 0.70(mol-S.A/mol-Gly), 12.78 (g/l) S.A. and 0.24 (g-S.A./l h) respectively on a medium containing initially 15 g/L glycerol. No cell growth was observed above 50g/l initial glycerol concentration. Although, these results are lower than 1,3-PD production from glycerol and succinic acid production from glucose, indicate the need for further investigation of this promising bio-process.

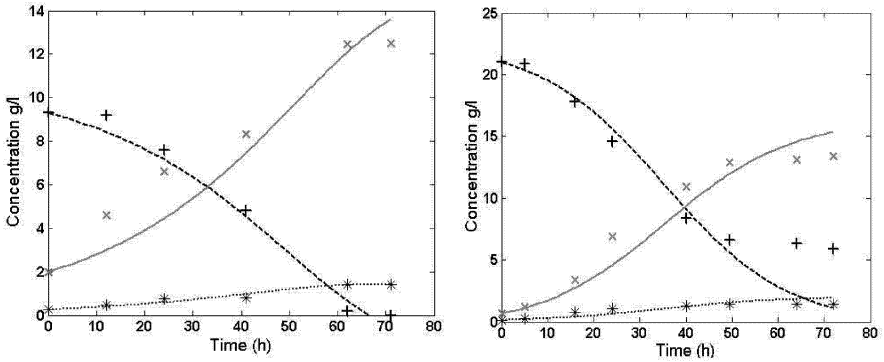

*Figure 1: Experimental and simulation data of succinic acid (solid lines, '**x**'), biomass (dotted lines, '*') and glycerol (dashed lines, **crosses**) for different initial glycerol concentrations and on different systems (left fig.: SAR initial glycerol concentration 9.3 g/l, right fig.: Bench-Top Reactor initial glycerol concentration 21 g/l)*

Table 2: Optimisation Parameters of the model

Parameters	Units	Fitted Values	Description	Bounds
μ_{max}	h^{-1}	0.71	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-P/g-X	4.67	Constant term for product	0.6-5.4
β	g-P/g-X h	0.001	Product maintenance coef.	0.001-1.2
Y_X	g-X/g-S	2.02	Stoichiometric Yield of X	0.4-2.8
Y_{SA}	g-P/g-S	0.018	Stoichiometric Yield of SA	0.001-1.2
m_s	g-S/g-X h	0.02	Specific maintenance coef.	0.001-0.8

4. 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 describes substrate and product inhibition and can adequately predict batch fermentations on both examined systems (SARs, Bench Top Reactors) for a range of initial glycerol concentrations. Experiments were carried out on the best environmental conditions indicated by a 2^3 factorial design. The model parameter values estimated were comparable with values found for similar systems. Further investigations are needed to optimise the bioprocess as fed-batch and continuous fermentations should improve the process significantly. Furthermore, detailed models that give insights into the intracellular reactions and metabolic phenomena need to be developed.

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