Effects of Metabolic Engineering on Downstream Processing Operational Cost and Energy Consumption: The Case of *Escherichia coli*'s Glycerol Conversion to Succinic Acid.

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ABSTRACT

BACKGROUND

Currently, the criteria for selection of gene targets in strains oriented to fermentation-based bioprocesses development have been largely based on the bioproduct yield, concentration or productivity. Nevertheless the concentration and purification of the product of interest strongly influences the final production cost. Then, a metabolic engineering-downstream coupling evaluation would be central when selecting the metabolic pathways candidates in the strain. Here, we evaluated the effects of the metabolic engineering an *Escherichia coli* strain in a succinic acid (SA) fermentation process using glycerol on the downstream process in terms of operational cost and energy consumption.

RESULTS

Three strain scenarios were selected using a bi-level linear optimization problem solved by MILP, and simulated in a transient fashion with dynamic flow balance

analysis considering both biomass and SA productivity. Our results show that the succinic acid productivity constitutes a central parameter when selecting the appropriate gene targets for deletion nonetheless the presence of organic acids in the downstream process and biomass growth rate. The lowest capital cost and period of recovery was obtained for the strain which provided the highest SA productivity.

CONCLUSION

Here, we proposed a metabolism-downstream coupled model that shows that the bioproduct productivity as the main element to contemplate when bearing in mind the operational cost and the energy consumption in the engineering of strains for industrial-scale production.

Keywords: *Metabolic engineering, Downstream, Process, Energy*

INTRODUCTION

Succinic acid (SA) has been constantly reported in the chemistry market as an important precursor participant in pharmaceutical, food, and chemical industries(1,2). Also, It can be used to generate value added products such as butyrolactone and 1, 4-butanedioic acid (2,3). There exist different strategies oriented to obtain it with high productivity yet low cost. Among them, petrochemical industry is nowadays the one who offer the most cost-effective alternative becoming the major difficulty for the bioprocess-based approach. Regarding the strains capable of producing the acid from an industrial perspective, there have been a lot of efforts to obtain high productivity strains based on bioprospecting and metabolic engineering. Wild strains include *Actinobacillus succinogenes*(4–6), *Anaerobiospirillum succiniciproducens* (7) and *Mannheimia succiniciproducens* (1) while genetic engineering efforts have been mainly

emphasized on working with *Escherichia coli* (*E. coli*) (8–10) and *Saccharomyces cerevisiae* (11). *E. coli* has been the most studied because of the knowledge for carrying out heterologous expression and deletion(12).

Table 1. Bio-based production of SA by metabolic engineering.

Microorganism	Deletions	Overexpression	Carbon source	SA obtained	Ref
<i>Escherichia coli</i>	<i>adhE</i> , <i>pta</i> , <i>ldhA</i> , <i>poxB</i> , <i>pflB</i>	Pyc	glycerol	0.69 g succinate / g glycerol	(8)
<i>Escherichia coli</i>	<i>sdhAB</i> , <i>iclR</i> , <i>poxB</i> , and <i>ackA-pta</i>		glucose	1 mol succinate /mol glucose	(13)
<i>Escherichia coli</i>	<i>tdcE</i> , <i>ackA</i> <i>adhE</i> , <i>aspC</i> , <i>citF</i> , <i>focA</i> , <i>ldhA</i> , <i>mgsA</i> , <i>sfcA</i> <i>pck</i> , <i>pflB</i> , <i>poxB</i> , <i>tdcD</i>			0.77 g succinate / g glucose	
<i>Escherichia coli</i>	<i>ackA-pta</i>		glucose	1.71 mol succinate / mol glucose	(2)
<i>Yarrowia lipolytica</i>	<i>Ylsth5</i>		glycerol	0.4 g succinate / g glycerol	(14)
<i>Saccharomyces cerevisiae</i> .	<i>gpd</i> , <i>fum</i> , <i>pdc</i>		glucose	0.32 mol succinate / mol glucose	(15)
<i>Corynebacterium glutamicum</i>	<i>ldhA</i>	Pyc	glucose	1.40 mol succinate / mol glucose	(7)
<i>Actinobacillus succinogenes</i>		<i>pck</i> , <i>mdh</i> , <i>fum</i>	xylose	0.65, 0.71, and 0.67 g succinate / g xylose	(16)

SA synthesis can be carried out either aerobically or anaerobically by exploiting different pathways (Table 1). Anaerobically, carboxylation of oxaloacetate is necessary to produce SA (2) and this leads to the accumulation of acids such as lactate, acetate and formate based on a NADH unbalance (17). Metabolic engineering based on gene deletion has been utilized to overcome different challenges for increasing the productivity and they are classified on substrate transport enhancement, gene overexpression and deletion (12,18–22). Some of them include PTS phosphotransferase inhibition, cyanobacterial *ppc* overexpression and *sdhAB*, *poxB*, *ackA-pta*, *iclR* and *ptsG* mutant development (1,12,23,13). Trial and error and an

understanding of the main routes that play a big role on the synthesis have been the base of the rationality behind the selection of targets for increasing productivity. Nevertheless, the prediction of the phenotype has been a constant challenge because it is necessary to evaluate what are the implications on the viability of the cell when carrying out deletions. Systems biology has permitted to predict such phenotypes utilizing an *in silico* approach. The amount of genomics, transcriptomics, metabolomics and proteomics data has facilitated the reconstruction of metabolic networks in a genomic scale (24–27). Then, based on mass and energy restrictions and an established objective function, Constraint-Based Reconstruction and Analysis simulations are conducted which finally provide the distribution of metabolic flows inside the cell upon different scenarios including overexpression and gene deletion(28). These simulations could be carried out by performing flux balance analysis assuming steady or transient states with linear programming (LP) optimization for steady state and LP or non-linear optimization (NLP) (29–31) problems respectively as follows:

For steady state:

$$\begin{aligned}
 &\textbf{Maximize} && v_{cellular\ objective} \\
 &\textbf{Subject to} && \sum_{j=1}^m S_{ij}v_j = 0 \\
 &&& lb_j \leq v_j \leq ub_j
 \end{aligned} \tag{1}$$

Here, $V_{cellularobjective}$ is the objective function. S is a matrix that describes the metabolism. The size of the S matrix is m -by- n , where m is the number of metabolites, and n is the number of reactions on the model. S_{ij} is the stoichiometric coefficient of metabolite i in reaction j . v_j represents the flux of reaction j . The network is assumed to

be at quasi-steady-state represent by $S_{ij}v_j = 0$. The upper and lower bounds of these reactions are described by lb_j and ub_j respectively.

For unsteady state:

The ordinary differential equations from the mass balance are obtained for each metabolite:

$$\begin{aligned}\frac{dz}{dt} &= S_{ij}v_j X_{biomass} \\ \frac{dx}{dt} &= \mu X_{Biomass} \\ \mu &= \sum w_j, v_j\end{aligned}\quad (2)$$

Where Z is a vector of metabolite concentrations, $X_{biomass}$ is the biomass concentration, V_j the metabolite flux vector expressed in gDW, S_{ij} is the stoichiometric matrix, μ is the growth rate and w_i is the amount of growth precursors per gram of biomass. Therefore, Dynamic Flux Balance Analysis (DFBA) enables the simulation of dynamics of biological systems(32). The NLP optimization problem could be described as:

$$\begin{aligned}\textbf{Maximize}_{z(t), v(t), X(t)} \quad & \hat{w}_{end} \phi(z, v, X)|_{t=t_f} + \hat{w}_{ins} \sum_{j=0}^M \int_{t_0}^{t_f} L(z, v, X(t)) \delta(t - t_j) dt \\ \textbf{Subject to} \quad & t_j = t_0 + j \frac{t_f - t_0}{M}, \quad j = 0 \dots M \\ & c(v, z) \leq 0, |\dot{v}| \leq \dot{v}_{max}, \quad \forall t \in [t_0, t_f] \\ & z(t_0) = Z_0 \\ & X(t_0) = X_0\end{aligned}\quad (3)$$

Here Z_0 y X_0 are the initial conditions for each metabolite and biomass concentration, $c(v, z)$ is a vector function that represent nonlinear constrains only if kinetic expression

are considering for the fluxes, t_0 y t_f are the initial and final time, ϕ is the objective function in the final point which is dependent of the final concentration in the final point, L is the snapshot objective function, δ is a Dirac Delta function, t_j is the instant of time where L is considered, $\hat{w}_{ins}$ y $\hat{w}_{end}$ are weight associated to L and ϕ and $v(t)$ is the metabolite profile in the time. Moreover, the equations 2 are considered as constraints for this problem. This problem is solved by parametrizing the dynamic equations through in the use of orthogonal placement of finite elements. The period of time t_0 y t_f is divided into a finite number of intervals. Flows, metabolite levels and biomass concentration, are parameterized in the roots of an orthogonal polynomial within each element finite. Continuity of the metabolite and concentration at the start of each finite element is imposed, the dynamic equations are transformed into algebraic equations.

Table 2. Comparison of different studies regarding recovery and purification of succinic acid.

Unit operations	SA extracted	SA concentration	SA purity	Ref
Reactive extraction, vacuum distillation, crystallization.	--	--	99.8%	(33)
Extraction by two aqueous phases using Ionic Liquid and water-miscible alcohols /sales/sales, distillation , crystallization.	94.4%	--	98.7%	(34)
Electrodialysis conventional, electrodialysis membrane stacks	-	From 51.5% to 79.6%	--	(1)
Precipitation of succinate salts	-	--	From 44.5% to 99.2%	(1)
Integrate fermentation and membrane ultrafiltration, absorption, distillation and crystallize.	85-90%	99.4%	99.4%	(35)
Electrodialysis, Nanofiltration, and Donna dialysis.	--	--	76%	(36)
Separation for two liquid phases	46%	--	--	(37)
Salting-out extraction, crystallization	--	--	97%	(38)
Liquid–liquid extraction using a hollow fiber membrane contactor	77%	--	--	(39)

Metabolic engineering of the strain provides only the information regarding the SA phenotype; nevertheless, it is necessary to consider the production cost and operability of the process in a large scale. Downstream processing in SA production covers 50-60% of the total production cost (33,40). Therefore, a wise selection of the strategy and the operation variables for the purification process is a key factor in the effort to achieve a cost-effective process. The high cost in the recovery and purification processes is associated with physicochemical properties shared with other carboxylic acids such as lactic acid, citric acid, acetic acid, oxalic acid, and formic acid (3,41,42). Previously mentioned typical fermentation acids can be accumulated when metabolic engineering is applied to the cell. Different schemes and unit operations have been proposed for the purification of SA. A general recovery and purification process of SA involves cell removal, liquid-liquid extraction, adsorption, and precipitation, conventional electrodialysis, distillation or crystallization. Some authors have evaluated the recovery of succinic acid and they have reported several alternatives (Table 2). Then, a coupling analysis of the downstream process and the metabolic engineering targets is required to determine the right approach to evaluate the production cost. Here in this work, we propose an *in silico* approach aimed to determine what are the effects of the SA productivity when obtaining succinic acid from glycerol using *E. coli* upon three different metabolic engineering scenarios on the production cost.

MATERIALS AND METHODS

Here we evaluated three different scenarios predicted from the network aimed to determine the effect on the operation cost and energy consumption. First, we carried out an integer optimization approach in order to determine potential *in silico*

knock outs (30). Secondly, we predicted the concentration profiles departing from the previously mentioned deletions as constraints with dynamic flux balance analysis (DFBA). Finally, a SA purification process was simulated and operational cost and energy consumption were obtained. The glycerol bioconversion to succinic acid was evaluated using the *E. coli* core model reported by Hädicke O, and Klamt S (43) which involves the central metabolism including reactions for the glycerol consumption. The batch reactor simulation was carried out using GAMS (General Algebraic Modeling System, GAMS Development Corp., Washington, DC). Linear Programming (LP) and Mixed Integer Linear Programming (MILP) was developed with solver CPLEX 12.6.0.0. DFBA provided the data for the downstream processing simulation using Aspen Plus® (Aspen Technology Inc., USA) V9.0. The recovery and purification processes were simulated with a set operation time of 8,766 h/yr. The processing operational cost was calculated using Aspen Process Economic Analyzer® (Aspen Technology Inc., USA) V9.0. The energy analysis was carried out by Aspen Energy Analyzer® (Aspen Technology Inc., USA) V9.0.

Metabolic engineering targets prediction

Gene deletion targets were obtained using a bi-level linear optimization problem solved by MILP (30). This strategy searches for a set of reactions maximizing the flux of a chemical objective while maximizing the biomass objective using the duality theory represented by the following equations:

$$\textbf{Maximize} \quad v_{\text{chemical objective}}$$

$$y_j$$

$$\textbf{Subject to} \quad \textbf{Maximize} \quad v_{\text{biomass objective}}$$

$$\begin{aligned}
& v_j \\
\textbf{Subject to} \quad & \sum_{j=1}^m S_{ij} v_j = 0 \\
& v_{pts} + v_{glk} = v_{gly_uptake} \\
& v_{atp} \geq v_{biomass}^{target} \\
& lb_j * y_j \leq v_j \leq ub_j * y_j \\
& y_j = \{0,1\} \\
& \sum_{j \in M} (1 - y_j) \leq K \tag{4}
\end{aligned}$$

Where, S_{ij} is the stoichometric matrix, v_j represent the flux reaction, v_{gly_uptake} is the basis glycerol uptake scenario, v_{pts} and v_{glk} are the uptake of glucose through the phoshphotransferase system and glucokinase respectively, $v_{biomass}^{target}$ is a minimun level of biomass production, lb_j and ub_j are the limits for each reaction, y_j is the binary variable. The primal problem is described such as maximizing a grow objective and a dual associated described such as ATP maintenance, biomass, and glycerol. For its part, deletions are represented for the incorporation of a binary variable, which is represented as 1 for active fluxes and 0 for inactive ones. Then, the set of active reactions assume any value between a lower and upper bound identifying a final gene/reaction knockout, which based on the Gene-Protein-Reaction (GPR) relationship could result in gene deletions.

Dynamic flux balance analysis for obtaining concentration profiles in the reactor

For the batch reactor simulation, the central *E. coli*'s metabolism represented in 486 metabolites and 499 reactions and a minimal media M9 were considered(44). The concentration profiles of Succinate, Acetate, Lactate, Formate, and Ethanol were obtained from a DFBA. This approach is an extension of FBA (45–48) which applies flux distribution constraints allowing to characterize dynamic concentration profiles modeling a batch fermentative process by incorporation of constraints. DFBA involves the optimization in a specific time interval to obtain concentration profiles of metabolites therefore allowing to perform simulations for the downstream process. Here, we used a Static Optimization Approach of DFBA (46). SOA optimizes for a specific time (36 hours) which is divided in N time intervals solving an LP optimization problem for each time interval by integrating over the interval represented as:

$$\underset{v(t)}{\text{Maximize}} = \sum w_i v_i(t)$$

$$\text{Subject to } z(t + \Delta T) \geq 0 \quad v(t) \geq 0$$

$$\hat{c}(z(t))v(t) \leq 0 \quad \forall t \in [t_0, t_f]$$

$$|v(t) - v(t - \Delta T)| \leq \dot{v}_{max} \Delta T \quad \forall t \in [t_0, t_f]$$

$$z(t + \Delta T) = z(t) + Av\Delta T$$

$$X(t + \Delta T) = X(t) + \mu X(t)\Delta T \tag{5}$$

Here $c(v, z)$ is a vector function that represents nonlinear constraints only if kinetic expressions are considered for the fluxes, ΔT is the length of the time interval chosen. The dynamic equations are integrated assuming that the fluxes are constant over the

interval. The optimization problem is formulated in the next instant of time and is solved repeatedly since t_0 until t_f . The *in silico* deletions were simulated by blocking the flux of the reactions in the DFBA predicted by the previous mentioned MILP problem.

Downstream processing for succinic acid

The results provided at t_f in the DFBA were used to describe the input for the downstream process. A purification model was developed to evaluate the recovery and purification process of SA by a direct evaporation process (Figure 1). Required physicochemical data (vapor pressure, solubility, etc.) were obtained from de Aspen Plus® V9.0. The nonrandom two liquid (NRTL) thermodynamic model was utilized to calculate the activity coefficients in the liquid phase and the Hayden-O'Connell (HOC) equation of state was used to model the vapor phase. The selected conditions for the fermentation (stream 1) and sulfuric acid stream (stream 2) were 1 bar of pressure and 37°C - 25°C, respectively. The SA purification process begins with an acidification. The conversion of carboxylates (succinate, acetate, lactate, and formate salts) to carboxylic acids is carried out using sulfuric acid (49) at 25°C and 1 bar. Then, an evaporator that vaporizes a large portion of the water is considered. Subsequently, a crystallization process was used for SA based on the solubility data at a temperature of 4°C. Finally, a liquid-solid separation using continuous rotatory vacuum filter is used to recover the succinic acid crystal (50)(Figure 1).

Figure 1. Flowsheet of succinic acid recovery and purification processes by a direct evaporation process. Stream composition can be observed in the supplementary data 2-4.

Aspen Plus® (Aspen Technology Inc., USA) optimization algorithm was utilized aimed to maximize the mass flow of SA crystal (stream 9 in figure 1). Sulfuric acid mass flow, heater, flash, cooler, and crystallizer temperature (Figure 1) were modified to optimize this flow (51).

RESULTS AND DISCUSSION

Gene deletion targets prediction.

A maximum of twenty reactions and 20 gene knockouts were considered for each prediction. A combinatorial of twenty six reactions were predicted finding Fumarase and alpha-ketoglutarate dehydrogenase as important keys in the Tricarboxylic Acid (TCA) cycle for the control of the SA production in the cell (1) (Figure 2). Three *in silico* designs were considered according to the biomass and succinate flux obtained (Table 1 and figure 2). These variables were selected in order to consider both productivity and yield effects on the downstream. For the strain 3, synthesis of acetate, lactate, formate, and ethanol was considered as mandatory in the prediction by the reaction

knockouts of PFL, LDH_D, POX, PTAr, and ACALD (Figure 2 and Table 3) in order to consider an important cost factor in the separation process(41,52).

Table 3. Biomass and chemical yields for various gene knockout strategies**.

Reaction Knockout	Id Reaction	Enzyme	Biomass (1/hr)	Succinate (mmol/gDW hr)
WildType				
Complete Network			0.5633	0.0000
Strain 1				
'ac_c + atp_c <=> actp_c + adp_c '	ACKr	'Acetate kinase'	0.3068	2.7534
'fum_c + h2o_c <=> mal__L_c '	FUM	'Fumarase'		
'6pgl_c + h2o_c -> 6pgc_c + h_c '	PGL	'6-phosphogluconolactonase'		
'dhap_c <=> g3p_c '	TPI	'Triose-phosphate isomerase'		
Strain 2				
'akg_c + coa_c + nad_c -> co2_c + nadh_c + succoa_c '	AKGDH	'2-Oxoglutarate dehydrogenase'	0.0576	6.0772
'6pgc_c -> 2ddg6p_c + h2o_c '	EDD	'6-phosphogluconate dehydratase'		
'atp_c + glyc__R_c -> 3pg_c + adp_c + h_c '	GLYK	'Glycerol kinase'		
'mal__L_c + nad_c <=> h_c + nadh_c + oaa_c '	MDH	'Malate dehydrogenase'		
'mal__L_c + nad_c -> co2_c + nadh_c + pyr_c '	ME1	'Malic enzyme (NAD)'		
'mal__L_c + nadp_c -> co2_c + nadph_c + pyr_c '	ME2	'Malic enzyme (NADP)'		
'coa_c + nad_c + pyr_c -> accoa_c + co2_c + nadh_c '	PDH	'Pyruvate dehydrogenase'		
'h2o_c + pyr_c + q8_c -> ac_c + co2_c + q8h2_c '	POX	'Pyruvate oxidase'		
'accoa_c + pi_c <=> actp_c + coa_c '	PTAr	'Phosphotransacetylase'		
'r5p_c + xu5p__D_c <=> g3p_c + s7p_c '	TKT1	'Transketolase'		
'dhap_c <=> g3p_c '	TPI	'Triose-phosphate isomerase'		
Strain 3				
'acald_c + coa_c + nad_c <=> accoa_c + h_c + nadh_c '	ACALD	'Acetaldehyde dehydrogenase (acetylating)'	0.1089	5.5661
'akg_c + coa_c + nad_c -> co2_c + nadh_c + succoa_c '	AKGDH	'2-Oxoglutarate dehydrogenase'		
'glyc3p_c + nadp_c <=> dhap_c + h_c + nadph_c '	G3PD2	'Glycerol-3-phosphate dehydrogenase (NADP)'		
'glyc_c + nad_c -> dha_c + h_c + nadh_c '	GLYCDx	'Glycerol dehydrogenase'		
'6pgc_c + nadp_c -> co2_c + nadph_c + ru5p__D_c '	GND	'Phosphogluconate dehydrogenase'		
'lac__D_c + nad_c <=> h_c + nadh_c + pyr_c '	LDH_D	'D-lactate dehydrogenase'		
'mal__L_c + nad_c <=> h_c + nadh_c + oaa_c '	MDH	'Malate dehydrogenase'		
'mal__L_c + nad_c -> co2_c + nadh_c + pyr_c '	ME1	'Malic enzyme (NAD)'		
'mal__L_c + nadp_c -> co2_c + nadph_c + pyr_c '	ME2	'Malic enzyme (NADP)'		
'coa_c + pyr_c -> accoa_c + for_c '	PFL	'Pyruvate formate lyase'		
'h2o_c + pyr_c + q8_c -> ac_c + co2_c + q8h2_c '	POX	'Pyruvate oxidase'		
'accoa_c + pi_c <=> actp_c + coa_c '	PTAr	'Phosphotransacetylase'		
'dhap_c <=> g3p_c '	TPI	'Triose-phosphate isomerase'		

**abbreviations for each metabolite are explained in the supplementary data 1.

We found that our simulations predict similar results compared to the reported engineered strains aimed to increase SA production from several carbon sources in *E. coli* ATCC 8739 and *E. coli* K12 (Table 1) (8,12,19,13,53–56). Also, in a study developed by Jantama, *et al* (2008) (2) the genes and enzymes considered: *citDEF* (citrate lyase), *gltA* (citrate synthase), *aspC* (aspartate aminotransferase), *ASPA* (ammonium lyase aspartate (malate)), *ppc* (phosphoenolpyruvate carboxylase), *sfcA* (NAD + -linked malic enzyme) which are part of the decarboxylation during gluconeogenesis, FUMA and *fumB* (fumarase), *frdABCD* (fumarate reductase), Pyka and *pykF* (pyruvate kinase), *tdcE* (pyruvate formate-lyase (similar to *PflB*)), *pta* (phosphate acetyltransferase) and TCDD (kinase propionate (ackA counterpart)) are similar to our findings with the model (57). Then we believe that the posed optimization problem provides results that allowed to continue with the prediction of the profile of metabolites of interest using DFBA.

Figure 2. Graphical representation of the *E. coli* core model 2(43). Crosses represented the reaction predicted for the gene deletion predictor, Reactions names are given in blue, except for the extracellular reactions. A. strain 1. B. strain 2. C. Strain 3, Green crosses were considered as mandatory reactions for the strain 3.

Concentration profile in batch reactor

Three engineered strains were considered to evaluate the concentration profile (Table 1). Lower growth rates 0.0578 and 0.1089 in the strain two and three, respectively, were obtained compared with strain one. These low growth rates have also been observed in other studies considering a significant amount of deletions (8,18,54,58). The observed total growth time was 60 hours for the strain 2 and 37 hours for the strain 3 (Figure 3) being a potential negative factor for the productivity of the process.

Figure 3. Biomass production, glycerol, succinate, and by-products concentration profile during batch fermentation. A. wild-type strain. B. Strain 1. C. Strain 2. D. Strain 3.

The batch fermentation process was simulated for a culture using 10 g/L of glycerol under aerobic conditions. A significant difference (around 50%) was observed for SA concentration was obtained for the strain 1 and the strain 2 and 3. The inlet stream for the downstream contains succinate and acetate for the three engineered strains and the strain with eleven knockouts containing restrictions for the elimination of acetate, lactate, formate, and ethanol, provided lactate (strain 2). The obtained lactate concentration was similar to the obtained for succinic acid, 6,09 g/L, and 7,66 g/L, respectively. These results are comparable with the results described by Jantama,

et al (2008) (2), who reported that succinate and acetate are the main products from fermentations.

Downstream processing and economics

The inlet stream for the downstream process was provided by the DFBA simulations. The total mass flow for the stream 1 (Figure 1) was fixed to 100000 kg/h as previously reported (40). The conversion of the reactor was obtained after optimizing the operation variables including the sulfuric acid stream (Table 4). A large portion of water and acetic acid is separated during the evaporation. Moreover, regarding the strain 2, 40% of the lactic acid was evaporated due to the boiling point for this compound. Subsequently, it was necessary to decrease the temperature of this stream down to 4°C for the crystallization (60). Regarding the Strain 2, we assumed that the lactic acid that enters the filter was water-miscible hence it does not crystallized as SA (59). For these models, the biomass removal was not evaluated in order to elucidate the effects of the presence of organic acids on the separation (40). We found significant differences in the sulfuric acid consumption due to the presence of different acids for those strains which provided the highest SA productivity (Strain 2 and 3), moreover, the presence of more acids in the stream demands an increase in the heater temperature which could affect the energy consumption during the operation.

A cost analysis was carried out for the three strains (Table 5). The lowest capital cost and period of recovery was obtained for the strain 2 which provided the highest SA production per year clearly demonstrating that when engineering a strain the most important factor to consider is the strain's productivity regardless of the presence of different by-products such as lactate, ethanol, or acetate that negatively affect the downstream operational cost as the sulfuric acid stream and heater temperature

increases. We did not find significant differences for the SA recovery demonstrating that the presence of different acids can be solved upon manipulation of the operational variables selected. On one hand we found that strains two and three displayed SA annual sales values larger than the annual costs, and on the other hand, SA annual sales are equal to the annual costs for strain one. Even though operational cost covered the most part of the cost for the production (Figure 4) (49) (59), our results indicate that SA productivity plays the most important role since the cost perspective.

Table 4. SA production and optimized variables for Strains 1, 2 and 3.

	Strain1	Strain2	Strain3
SA production per year (x106kg/yr)	2,8	6,2	5,8
SA recovery (percentage)	92,0	92,2	94,9
Optimized Variables			
Sulfuric acid inlet stream to reactor (kg/h)	444,1	985	595
Heater Temperature (°C)	72	80	80
Flash Temperature (°C)	109,1	110	110
Cooler Temperature (°C)	4	4	4
Crystallizer Temperature (°C)	0	0	0

Table 5. Economic Analysis for the SA downstream processing.

	Strain1	Strain2	Strain3
Total Capital Cost [USD]	3.800.680	3.415.770	3.399.520
Total Operating Cost [USD/Year]	3.902.660	6.419.670	5.150.990
Total Raw Materials Cost [USD/Year]	882.589	2.935.180	1.773.030
Total Product Sales [USD/Year]	6.306.230	13.624.800	12.848.300
Total Utilities Cost [USD/Year]	1.528.670	1.798.930	1.787.020
Desired Rate of Return [Percent/Year]	20	20	20
P.O. Period [Year]	4,84	1,91	1,71
Equipment Cost [USD]	460.000	453.900	445.900
Total Installed Cost [USD]	1.027.300	876.700	865.400

*The price considered for the sulfuric acid was the 0.034 USD/kg and 2.2 USD/kg for succinic acid.

Figure 4. Downstream processing annual costs for each strain predicted.

Table 6: Energy consumption from SA purification process for Strains 1, 2 and 3.

	Strain 1	Strain 2	Strain3
Total Utilities (Gcal/hr)	93.74	61.78	62.05
Heating Utilities (Gcal/hr)	92.54	61.62	61.62
Cooling Utilities (Gcal/hr)	1.2	0.1637	0.4327

Energy cost associated with cooling and heating utilities

Energy savings were calculated for each metabolic engineering option (Table 6). Even though strain 1 displayed the highest biomass production rate which should be reflected on the energy consumption savings, our results indicated that the SA productivity is the factor which finally decides which strategy is the most favorable the economics and the energy point of view. Heating and cooling utilities cost displayed a 30 % decrement for strains two and three compared to strain one and this was finally reflected on the recovery period (Table 6). Also, in this work was observed that the energy expenditure for the three strain predicted, was around twice for strain 1 in contrast with the strain 2 and 3.

Conclusions

Here, by an *in silico* approach, we developed a model for understanding the potential effects of the decisions behind engineering strains on the separation cost and energy

consumption utilizing a *E. coli* model and the production of succinic acid using glycerol. By coupling the fermentation model with the purification process we found that the factor which plays the biggest role on the economics of the process is the SA productivity regardless of the influence of the stream composition taking into account the presence of different organic acids and the biomass growth rate of the strain. Moreover, the operational cost associated with the purification was found to be strongly coupled with the process economics because the selection of the appropriate genes to knock out taking into account the energy consumption is based on SA productivity

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