STUDIA UBB CHEMIA, LIX, 2, 2014 (p. 33 - 50)
(RECOMMENDED CITATION)

BIOTECHNOLOGICAL PRODUCTION OF SUCCINIC ACID FROM GLYCEROL; THE ROLE OF CO-SUBSTRATES

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ABSTRACT. Our society is merely based on petroleum, however its availability and the impact on environment makes this energy source unsustainable. Hence, search for new renewable energy sources is of major interest. Biodiesel as biofuel is a promising source and intensively studied for the substitution of fossil fuels. However, during the production process glycerol is generated as the main by-product (10% (w/w)). The bio-based conversion of glycerol to valuable chemical such as succinic acid by commonly used microorganisms like *Escherichia coli* is one of possible applications. Succinic acid is used in a number of industries including: polymers, food, it can be converted to biodegradable plastics, etc. Genetically engineered strains can be used to provide a cost-effective, ecologically sustainable alternative to the current petrochemical production process. The main aim of this study was to make predictions and to analyse the production of succinic acid from glycerol if different chemicals (co-substrates) are present in the minimal media. We found that the presence of co-substrate in minimal medium is critical under anaerobic conditions; on the other hand, with genetic modifications the succinic acid production can be significantly influenced. The *in silico* studies presented here may serve as important contribution to the implementation of biorefineries by converting biofuel waste glycerol, into a higher-value chemical, reducing environmental impacts. The new application of glycerol may improve the economic viability of the biodiesel industry.

Keywords: *Glycerol; Succinic acid; Escherichia coli; Metabolic engineering - Modelling;*

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INTRODUCTION

Petroleum is the main fossil energy source utilized worldwide, but the associated environmental impacts; climate change, air pollution, soil degradations, the diminishing of fossil fuel deposits have been raised many concerns, including sustainability [1]. Sustainable development is environment-connected and need to be harmonized to sustain a continuous development and hence, the applications of (bio-based) green technologies is crucial. One of the strong candidates in the conventional diesel engine is the biodiesel production, which is increasing year by year (in 2010 was 1.30 and in 2012 near 3.67 million m^3 in the USA) [1]. Biodiesel production process generates large quantities of glycerol (10% (wt/wt)) which could be an inexpensive carbon source for many microorganisms [1-3] and can be used as un appealing substrate for high value-added biochemicals production [4, 5]. Glycerol is a non-toxic liquid, however, like most organic materials, deplete the oxygen content of water and wetlands very quickly and can suffocate fish and other organisms. Birds can be deadly affected (crude oil spill) and on the other hand the use of rivers for fishing, boating, etc. can be seriously damaged [6].

Glycerol can be converted biologically to succinic acid, what is a dicarboxylic acid with four carbon atoms. It is an important precursor (building-block) for many industrially manufactured chemical commodities and products. For example, it can be hydrogenated into 1,4-Butanediol [7], a potential petroleum alternative, which can be further used to modify succinic acid to form the inexpensive biodegradable plastic polybutylene succinate [8]. A few applications are presented below [9] (Fig. 1).

The U.S. Department of Energy identified succinic acid as one of the 12 top chemical building blocks produced by microorganisms [10]. Today most of the succinic acid derives from the petrochemical industry, and the starting point is the non-renewable fossil fuel butane [11]. One possible way to reduce the “ecological footprint” is to create economically competitive biorefineries to produce fuels (e.g. biodiesel), chemicals (e.g. succinic acid) and different bio-products, reducing the petroleum dependency. The most important challenges is to use renewable resources; e.g. by-products generated by these industries and transform them biologically (using different microorganisms) into valuable chemicals such as succinic acid. To efficiently produce succinic acid biologically various attempts have been made such as, natural succinic acid producers as well as metabolically engineered strains [3, 11, 12].

Is crucial to grow the cell in a low cost medium and the engineered cell should be able to grow and produce the biochemical on the cheapest available raw material (waste-glycerol). The well known *Escherichia coli* could be a strong candidate because it is widely used in biotechnology and the necessary molecular methods are available to carry out genetic and metabolic modifications. The level of succinic acid produced by native strains of *E. coli* in minimal medium is very low [3]. To improve cellular capabilities and the production yield metabolic engineering should be carried out.

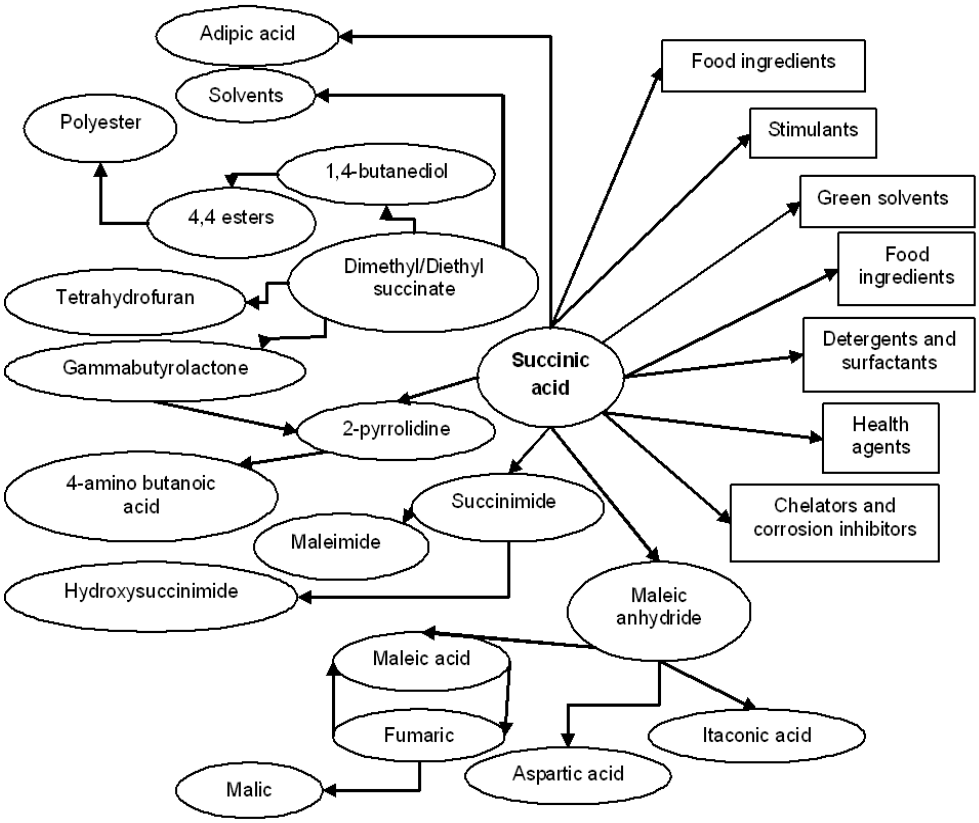

Figure 1. Value-added products that can be derived from succinic acid

Many scientists have described genetic engineering approaches to improve the production in *E. coli* [2,3,13,14] but mediums and applied methods are too complex and complicated. One way could be to block pathways of competing metabolic products such as, formic acid, lactic acid, ethanol and leaving only the succinic acid pathway to achieve redox balance during glycerol utilization [15] in minimal media (M9). Redox balance is not important from glucose, meanwhile it is of central importance in case of glycerol fermentation in *E. coli* [15], will reduce the growth rate or even the biomass production will be inhibited. To overcome these problems different co-substrates need to be used together with glycerol to improve cell viability.

Before *in vivo* tests is mandatory to *in silico* design the new strains, to analyse the behaviour under different genetic and environmental manipulations and to predict the genetic modifications impact on cell metabolism and to the desired flux distribution.

To analyse *in silico* large-scale biological networks, make predictions about cellular behaviours and test the impact or perturbations such as gene deletions, Flux Balance Analysis is an effective tool [16]. The biological models are constructed based on the known stoichiometry of the metabolic reactions, thermodynamic constraints and flux capacities [17]. One of the modelling platforms is COBRA Toolbox [18,19] an open-source and modular platform (widely used in systems biology), incorporating strain optimization tasks, algorithms such as: (FBA), dynamic FBA, phenotypic phase plane analysis, etc.

In this paper we present *in silico* and *in vivo* metabolic engineering studies with the model organism *E. coli* to obtain succinic acid under anaerobic conditions from glycerol. The main aim of the study was the identification of co-substrates necessary to improve the genetically engineered cell viability on glycerol using a complex genome scale metabolic model of *E. coli* [20] and to determine the dynamics of processes. First, we calculated the flux distributions, than we eliminated the three most widely knocked out genes in metabolic engineering (pyruvate formate lyase (*pflB*), lactate dehydrogenase (*ldhA*) and alcohol dehydrogenase (*adhE*)) – from pyruvate metabolism (to improve succinic acid production). The elimination of these genes (pathways) under anaerobic conditions is impossible without co-substrate. We analysed the fitness landscape using dynamic growth simulations, the correlation between genotype and phenotype and phenotypic phase plane analysis was carried out to determine the maximum growth while varying two parameters simultaneously. Wet experiments were carried out, to determine the growth rates and validate the model predictions.

A maximum yield of 0.50 (mol succinic acid mol^{-1} glycerol) can be achieved using glutamic acid or glutamine as co-substrates at an uptake rate of 1 (mM $gDW^{-1}h^{-1}$) (millimole per gram dry weight of cells per hour) using mutant strain. The change of co-substrate to glucose increased the yield to 0.60 (mol mol^{-1} glycerol).

With complex *in silico* studies we are able to design and genetically engineer industrially important strains, which can be future used in different biotechnological processes to convert renewable feedstocks such as, glycerol from biodiesel industry into a value added biochemical. It is clear that glycerol is a promising abundant carbon source and can improve the economic feasibility of biodiesel industry if will be used biologically, environmental impacts can be reduced and bio-based succinic acid can be obtained without petrochemicals.

RESULTS AND DISCUSSION

The main aim of this study was to redesign *E. coli* strain to create a mutant, being capable to produce succinic acid from renewable resource such glycerol in minimal medium under anaerobic conditions, to understand the succinic acid effect on growth rate and finally to test different co-substrates effect on cellular network. We decided to test co-substrates to enhance the cell viability, such as; aspartic acid, glutamic acid, alanine, glycine, glutamine and glucose (data not shown).

Succinic acid can be produced by *E. coli* under anaerobic conditions but the quantity of the excreted succinic acid is very low. As we know *E. coli* is able to utilize a variety of simple and complex carbohydrate substrate for growth in different environmental conditions.

Under aerobic conditions *E. coli* converts the substrates quantitatively to biomass and CO_2. Changing the environmental conditions (O_2 elimination) reduced biomass formation with 85% on glycerol. In anaerobic conditions the major metabolic by-product was formic acid followed by ethanol and acetic acid (Figure 2).

By-product pathways elimination

Succinic acid production from glycerol involves fixation of CO_2 (greenhouse gas reduction) onto a 3–carbon intermediate, which could be converted to succinic acid. With an uptake rate of 10 mM glycerol the maximal growth rate was 0.56 under aerobic conditions and 0.08 (h^{-1}) under anaerobic conditions (no significant differences were detected with co-substrates).

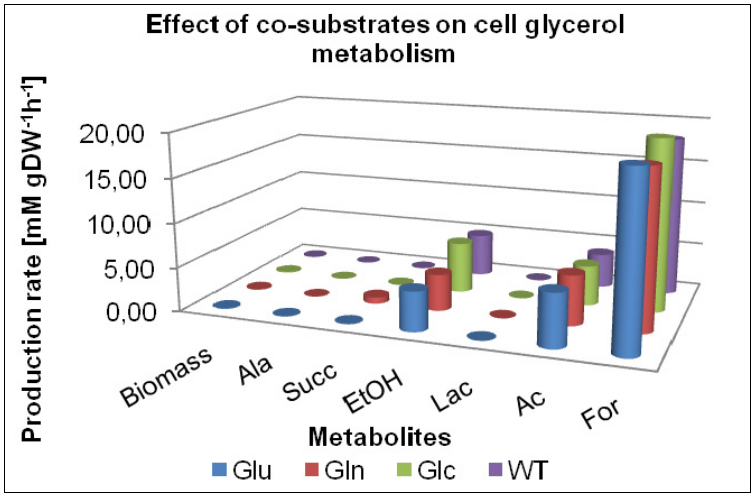

Figure 2. Production rate of different metabolites under anaerobic conditions using glycerol as carbon source and co-substrates; Glu: glutamic acid; Gln: glutamine; Glc: glucose; WT: wild-type without co-substrate; Ala: alanine; Succ: succinic acid; EtOH: ethanol; Lac: lactic acid; Ac: acetic acid; For: formic acid. Fluxes have units of mM $gDW^{-1}h^{-1}$, except for biomass, which has units of h^{-1}.

To increase the succinic acid production the mentioned genes were eliminated, but the mutant strain failed to grow anaerobically. The explication could be that Acetyl-CoA is an essential metabolite for biosynthesis that is produced primarily by *pflB* during fermentative growth [3]. Three best candidates were detected, two amino acids (glutamic acid, glutamine) and glucose.

As we can observe from (Figure 2) three major fermentation products were predicted in each cases as follows: formic acid, ethanol and acetic acid. It is clear that the elimination of formic acid is necessary because large amounts of carbon are lost. With co-substrates was possible to carry out the genetic modifications. The double mutant showed increased ethanol production. The third eliminated gene was the Δ*adhE* gene to block the synthesis of ethanol as by-product. The double mutant failed to produce succinic acid in a higher rate, but the elimination of the ethanol pathway had a positive effect on succinic acid production.

Using glutamic acid and glutamine as co-substrates at an uptake rate of 1 mM $gDW^{-1}h^{-1}$ and with the Δ*pflB, ΔldhA* and Δ*adhE* eliminations we obtained a succinic acid yield of 0.5 (mol mol^{-1} glycerol). On the other hand, using glucose as co-substrate the triple mutant succinic acid yield increased to 0.6 (mol mol^{-1} glycerol) with 1 mM $gDW^{-1}h^{-1}$ glucose uptake rates, however the cell growth rate was drastically reduced (0.001 h^{-1}) (Fig. 3).

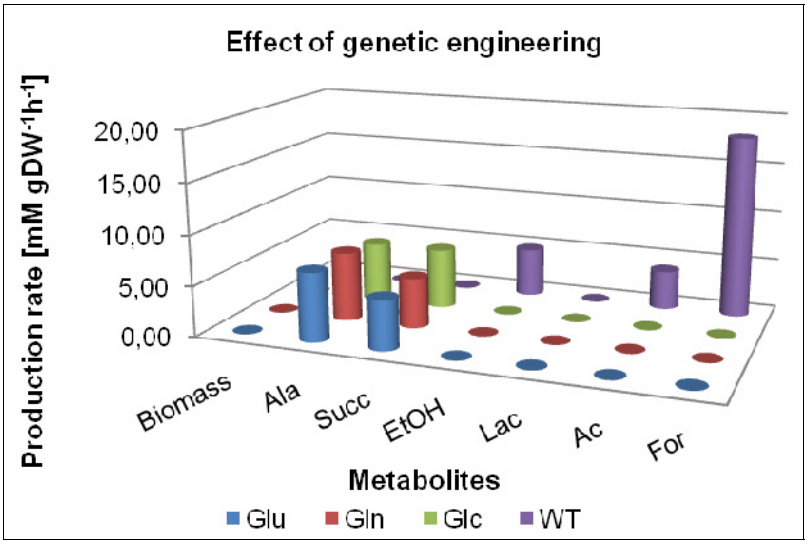

Figure 3. Production rate of different metabolites under anaerobic conditions using glycerol as carbon source and co-substrates after metabolic engineering

In each simulation the biomass production is negatively affected by the succinic acid production. Eliminating the pathways of the metabolites like formic acid, lactic acid and ethanol is considered as an ideal strategy to improve succinic acid yield from glycerol. In each case the production rate of alanine increased significantly compared to wild-type and the acetic acid production was inhibited.

Dynamic FBA of diauxic growth

Although classical static FBA is unable to predict the dynamics of metabolic processes, as the network analysis is based on steady-state solutions, time-dependent processes can be taken into account by extending the classical static FBA to a dynamic FBA (dFBA), as proposed by Schellenberger *et al*. 2011. The dFBA approaches were used to simulate batch growth of wild type and triple mutant (Δ*pflB,* Δ*ldhA,* Δ*adhE*) *E. coli* on glycerol using anaerobic conditions.

Dynamic FBA was performed to simulate batch growth in glycerol minimal media conditions with glycerol as the input and biomass, acetic acid, formic acid, ethanol, lactic acid and succinic acid as the outputs (Figure 4).

The growth rate of mutant strains is extremely low even with co-substrate under anaerobic conditions (Figure 4) without diauxic growth.

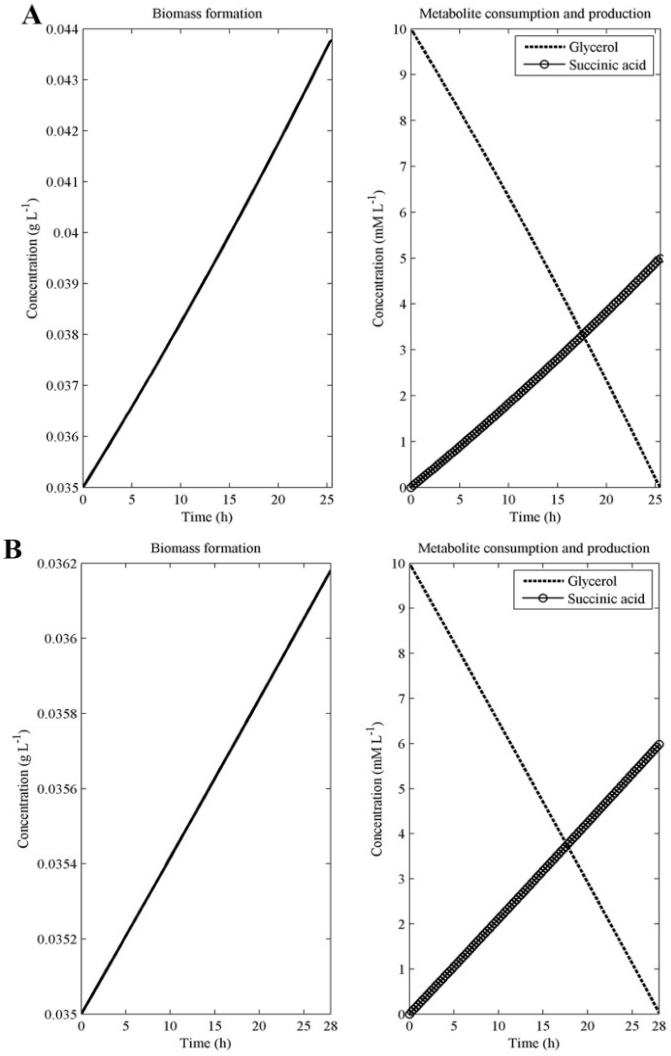

Figure 4. Model predictions using dynamic FBA for the outcomes of mutant strains; Gln (A) and Glc (B)

Phenotypic phase plane analysis (PhPP)

To analyse the optimal utilization of the wild-type and mutant *E. coli* metabolic genotype, phenotypic phase plane analysis was carried out for cellular growth *in silico* on glycerol substrate. We mapped the theoretical optimal metabolic characteristics for biomass production as a function of the environmental variables such as glutamine, glutamic acid and glucose as co-substrates and glycerol together with growth rates (Fig. 5).

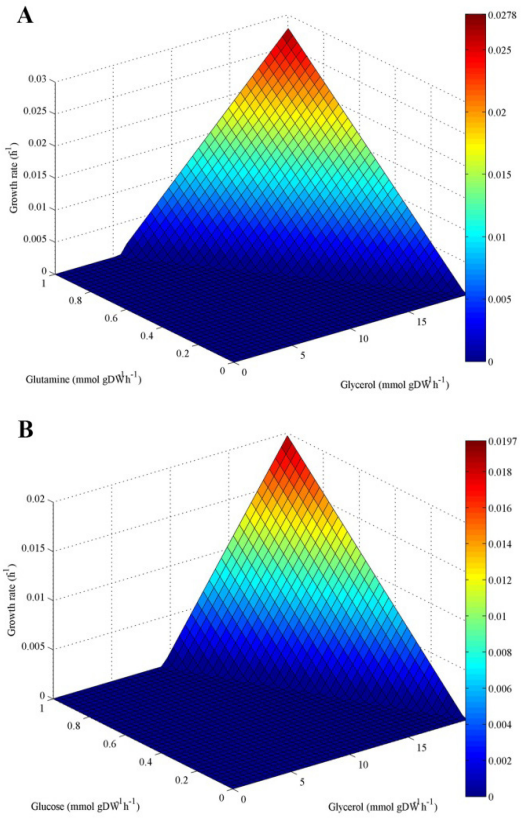

Figure 5. Phenotypic phase planes for growth of mutant strains: Gln (A) Glc (B)

It is clear from these plots that each surface has distinct regions, meaning qualitatively distinct phenotypes. The identified phenotypic phase planes are: 5 distinct regions for wild-type (not presented) and 2 for mutant strains with co-substrates (results are for glutamine and glucose co-substrates- there are no differences between glutamine and glutamic acid). Phase 1 (base plane) is characterized by 0 growths. By blocking important reactions in the NADH oxidation pathways the redox potential was significantly affected, led a different optimal metabolic phenotype.

Time-course fermentation experiments were carried out for wild-type and mutant strains (Δ*pflB, ΔldhA,* Δ*adhE*) to follow the changes of metabolites.

Metabolites were identified using GC-MS. Data analysis was carried out with MassLab via comparison with mass spectra obtained from different libraries. The GC chromatograms of the silylated metabolites from the fermentation mixtures are presented in Figure 6.

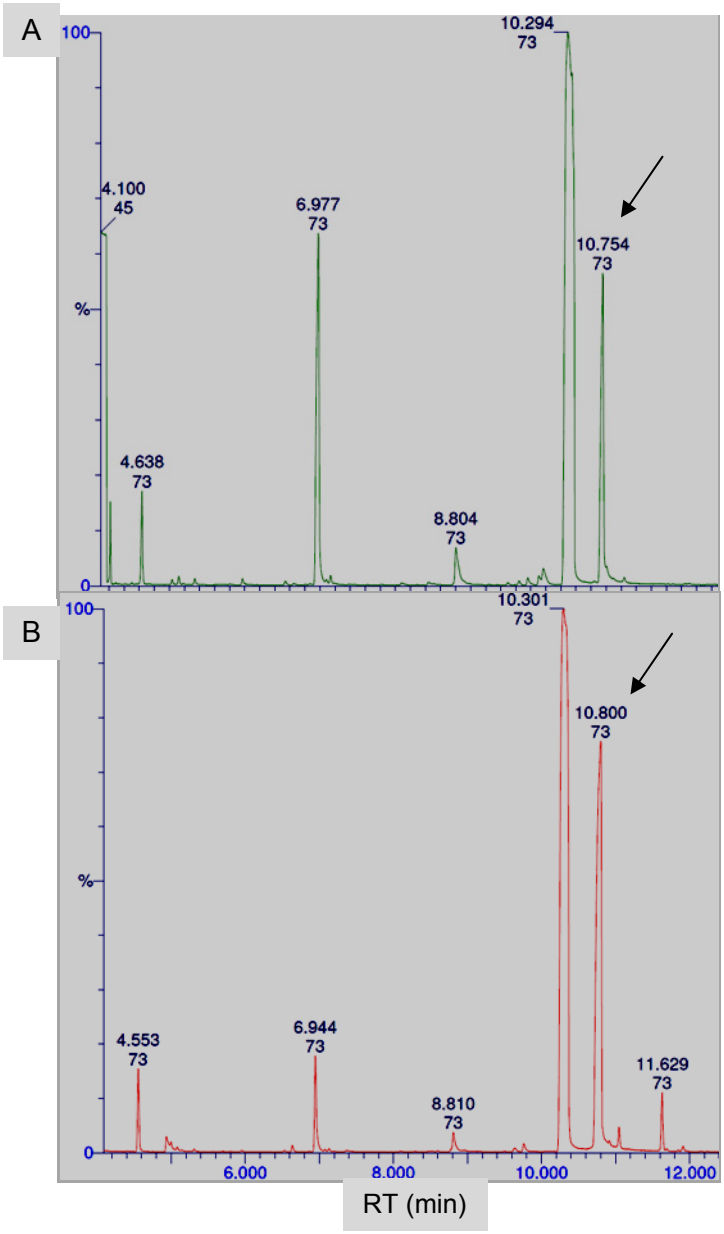

Figure 6. GC-MS analysis of determination of succinic acid from supernatants; wild-type (A), mutant with glucose as co-substrate (B); succinic acid peak was observed at 10’ 71”. Arrows indicates succinic acid peak.

The heights of the peak indicate the relative concentrations of the metabolites present in the fermentation mixtures. There was a good agreement between simulations and experiments taking into account the growth rates and succinic acid production rates.

CONCLUSIONS

In the long term demands for sustainable development and volatile petroleum prices should favour the use of greener chemicals even if their market price is not cheaper or sometimes even not competitive with the current processes. Increasing awareness of environmental issues and pressures from different public agencies to reduce the pollutions caused by petrochemical industry has led to the development of biomass conversion processes.

On the other hand, the microbiological conversion of by-products (wastes) generated by these methods seems to be the future direction. Important results were obtained in laboratory scale using yeasts and bacteria, especially with efficient selection and construction of recombinant strains based on biochemical and genomic data associated with optimization of fermentation conditions. Most widely considered is the well known *E. coli* with genetically engineered routes.

Biofuels role is critical in reducing green house gas (GHG) emissions and in the transition of the current petroleum-based society towards a more sustainable one. The biodiesel production cost is quite high, hence, the by-product conversion into value-added chemicals with fermentation will improve the net energy, sustainability and profitability of the biodiesel industry. Glycerol may be used as a starting material for large-scale biotechnological processes because it is a good energy and carbon source. Using specific conditions and co-substrates under anaerobic conditions it may be suitable for the biotechnological production of a number of chemicals in fermentative processes. Of course the success depends on strain and condition optimization to achieve the best results. *In silico* analysis are valuable tools to carry out cellular behaviour studies, design industrially important strains by genetic engineering and make predictions. We believe that in the near future the biorefinery concept will gain more attention and will be applied to large-scale processes.

We used the FBA method to investigate the network and global metabolic capability of *Escherichia coli* K12 MG1655. The flux distribution was

estimated under different environmental conditions and genetic modifications. Genome scale models are widely used to reconstruct metabolic networks, to obtain strains with increased capabilities, to produce important products like lycopene, succinic acid, lactic acid, etc. Bio-based succinic acid production has many advantages: raw material cost, increasing market size potential and carbon dioxide fixation.

The production of succinic acid from glycerol offers a green alternative. *E. coli* K12 strain MG1655 can ferment glycerol under anaerobic condition, but the elimination of pathways required a co-substrate to keep the cell viably. Three best candidates were found: glutamine, glutamic acid and glucose. With these genetic modifications the succinic acid yield was ~130 fold higher than in wild-type.

It is clear from these results that the perturbation of redox balance via the deletion of alternative, peripherial redox reactions can drastically affect the central mixed-acid fermentation. The current work demonstrated that the use of minimal medium and the disruption of three genes with co-substrates was sufficient to induce the overproduction of succinic acid from glycerol under anaerobic conditions.

Dynamic FBA was used to simulate batch growth in minimal media conditions to test the effect of co-substrates. Dynamic growth simulations and phenotype phase planes provides a deeper understanding of differences of the metabolic flux distributions between wild-type and genetically engineered strains, especially between genotype and phenotype. We can conclude that the role of co-substrates is crucial, but minimal changes were between them.

Growth rates were consistent during the experimentally measurements, the cultures (on glycerol- wild-type and mutant) were in the different regions on the phase planes; a few primary phenotypes were identified. Strikingly different phenotypes were found for mutant strains 2 PhPP compared to 4 in case of wild-type.

The λ-Red recombineering technology was successfully used for chromosomal modifications in *E. coli*.

The GC-MS was suitable for the identification and quantification of succinic acid.

The *in silico* strains design presented here may serve as important contributions to the implementation of biorefineries. The utilization of crude glycerol from biodiesel industry will improve the economic feasibility and a higher-value chemical (succinic acid) will be obtained reducing dependency on petrochemicals and the same time fixing CO_2 a well known green house gas.

EXPERIMENTAL SECTION

The reconstructed metabolic model of *E. coli* K12 MG1655 iJO1366 [20] was utilized as a basic model throughout the work described herein. The model is the most complex available functionally tested and verified against experimental data to predict correctly the growth rates, metabolites excretion rates and growth phenotypes under different substrate and genetic conditions [21]. The metabolic model is available in SBML format at BioModels online database [http://www.ebi.ac.uk/biomodels-main/]. The model contains 1366 genes, 2251 metabolic reactions and 1136 unique metabolites.

Flux balance analysis (FBA) for wild-type and mutant strains

The most popular constraint-based optimization approach is Flux Balance Analysis (FBA) a direct application of linear programming to biological systems. It was used for computing optimal phenotypes with the objective function of biomass production, using stoichiometric coefficients for each reaction, reversibility and fluxes constraints [22]. The objective function was the rate of biomass synthesis, as follows:

$$\text{Max: } v_{growth}=c^{T}*v, \quad (1)$$

where *c* denotes the vector defining the weights for each of the fluxes in *v.*

For mathematical representation of the stoichiometric matrix, *S*, is used (each column represents a reaction and each row a metabolite, the numerical elements are the stoichiometric coefficients). Steady-state metabolite flux assumption was performed for FBA calculations described in detail previously [23]. The lower and upper bounds of the fluxes (constraint addition):

- constraints $vl \le v \le vu$; *vl* and *vu* are vectors with *n* elements each, which represent the lower and upper bounds on the fluxes, respectively, the constraints for reaction irreversibility or substrate uptake from the environment. In this case the stoichiometric coefficients alone are sufficient for the mathematical maximization of a specific objective function [24].

All computations were performed in MATLAB (mathworks Inc.; Natick, MA, USA) using COBRA Toolbox (version 2.0.5, http://opencobra.sourceforge.net/openCOBRA/Welcome.html) (Becker et al., 2007) software packages with Gurobi optimization solver (Gurobi Optimizer version 5.1.0 Houston, Texas).

Computations

Simulations were carried out using minimal media (M9) containing only inorganic salts and for carbon source we used glycerol. As it is based on constraint based modelling at least one of the reactions must have an experimentally measured value (e.g. substrate uptake rate). One important differences between modelling and analyses of biological systems compared to methods used in physiochemical sciences is that in the former case the network is not stable and may have different states and solutions to reach the optimal value [25]. Consumption rate (substrate uptake rate) in each simulation was set to 10 mM $gDW^{-1}h^{-1}$- (millimoles dry cell weight per hour). The values are close to that observed experimentally for anaerobic growth in minimal media. To create anaerobic conditions the oxygen uptake rate was set to 0 mM $gDW^{-1}h^{-1}$.

Three reactions/pathways were eliminated by setting the upper and lower bounds to zero to analyse the cell behaviour. The eliminated reactions were: Δ*pflB*, Δ*ldhA* and Δ*adhE* (Figure 7).

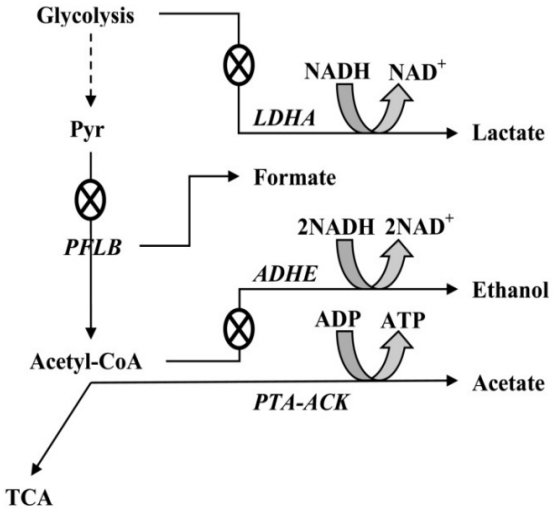

Figure 7. The genetic modifications carried out, crosses represent the reaction knockouts; the following pathways were eliminated: pyruvate formate lyase- Δ*pflB* (formate), lactate dehydrogenase-Δ*ldhA* (lactate), alcohol dehydrogenase-Δ*adhE* (ethanol); Abbreviations: Pyr, Pyruvate; *PFLB, Pyruvate formate lyase; Acetyl-CoA, Acetyl coenzyme A; TCA, Tricarboxylic acid cycle; LDHA, Lactate dehydrogenase; NADH*, nicotinamide adenine dinucleotide; *ADHE*, alcohol dehydrogenase; *ADP*, adenozin difosfat; *ATP*, adenozin trifosfat; *PTA-ACK*, phosphotransacetylase-acetate kinase.

Using the conditions mentioned before the *pflB* mutant failed to grow under anaerobic conditions. Different co-substrates were tested to improve the cell viability including glucose and amino acids.

Combining FBA with an iterative approach based on a quasi-steady-state assumption we are able to analyse dynamic processes e.g. growth rates, metabolites production and consumption rates [18]. The initial substrate concentration was set to 10 mM L^{-1} and the co-substrate uptake to 1 mM $gDW^{-1}h^{-1}$, while the initial biomass concentration was set to 0.035 g L^{-1} (~0.1 optical density (OD)). In order to observe if this diauxic growth is present or not time step was set at a higher value to 25 min and the maximum number of steps to 150 to allow the consumption of metabolites.

Phenotypic phase plane analysis (PhPP) was performed for wild type and mutant strains to identify the robustness of the system under different conditions if two parameters are varied simultaneously. Detailed description of this method can be found elsewhere [16].

Bacterial strain, culture conditions

The strain used in this study was *Escherichia coli* K12 MG1655 from "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ 18039). Fermentation was done at 37°C, in minimal medium under anaerobic conditions with glycerol at a concentration of 0.2% (v/v). Components of M9 medium per liter: $Na_2HPO_4*7H_2O$ (12.8 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), micronutrients final concentration: $MgSO_4$ (1 mM), $CaCl_2$ (100 µM), $(NH_4)6Mo_7O_{24}*4H_2O$ ($3*10^{-9}$ M), H_3BO_3 ($4*10^{-7}$ M), $CoCl_2*6H_2O$ ($3*10^{-8}$ M), $CuSO_4*5H_2O$ ($1*10^{-8}$ M), $MnSO_4$ ($8*10^{-8}$ M), $ZnCl_2$ ($1*10^{-8}$ M), $FeSO_4$-$7H_2O$ ($1*10^{-6}$ M). Bacterial cells were grown in minimal medium in 5 mL to produce a starter culture and the seed culture was used to inoculate the fermentation medium (OD=0.1). Cells were grown with shaking at 150 rpm (Certomat BS-1 Sartorius) for 24 h in serum bottles (50 mL) with 20 mL M9 medium. Samples were taken in every two hours for the analysis of cell growth. The optical density of the cell cultures was measured at 550 nm (OD_{550}) to quantify cell growth, using a Cary 50 Conc UV-Visible spectrophotometer, as well as dry cell weight determination. To estimate the cell mass we used the following simple assumption (1 OD_{550}=0.36 gDW L^{-1}). 1 mL of the culture supernatant was added in triplicate to pre-weighed Eppendorf tubes, centrifuged, washed with NaCl (0.9%) and dried until constant mass at 105°C.

Chromosomal gene deletion

For gene deletion strategy we used the λ-Red recombineering methods previously described [26]. Plasmids (5 Strain Wanner Lambda Red Gene Disruption Kit) were obtained from the *E. coli* Genetic Stock Center (Yale University).

Analytical procedure

Metabolites were analysed by *Gas chromatography coupled* to *mass spectrometry (GC-MS)* (6890N/5975 Agilent) based on solid-phase microextraction (SPME) with on-fiber silylation. Silylation was carried out using N, O bis (trimethylsilyl) trifluoroacetamide (BSTFA), following the procedures described elsewhere [27]. The relative % amount of succinnic acid was calculated by comparing its average peak area to the total areas, for chromatograms and spectra analyses we used the MassLab software (ThermoQuest, Manchester, UK). The identification of compounds were identified by comparing the mass spectra obtained with commercially available MS libraries (Wiley, NIST and LIBTX).

ACKNOWLEDGMENTS

This work was supported by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/107/1.5/S/76903, by “BIOBUILD-Synthesis of some C4, C5 carboxylic acid building block chemicals from renewable biomass resources“ PN-II-PCCA-2011-3.2-1367 and by Collegium Talentum. GC-MS analysis was assisted by *Center* for Organic Chemistry “Costin D. *Nenitescu*”.

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