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Faculty of Applied Chemistry and Materials Science
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THESIS

Metabolic engineering of Escherichia coli for obtaining 1,4-butanediol from glucose and glycerol

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Keywords: 1,4-butanediol , *Escherichia coli*, metabolic engineering, λ-Red recombination, heterologous expression, metabolic profile

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Contents

Objective and importance of the theme

1,4-Butanediol is an industrial chemical with good environmental characteristics, widely used in medicine, chemical, textile, paper making, automobile, and the daily-using chemical industries.

Possible applications and market potential of 1,4-butanediol is growing year by year, serving applications such as engineering polymers, performance elastomers and polymers, solvents and fine chemical intermediates.

The global production of BDO is currently manufactured entirely from petroleum-based, non-renewable feedstocks such as acetylene, butane, butadiene, etc. and 1,4-Butanediol is listed on the NICNAS High Volume Industrial Chemicals List (HVICL). The utilization of renewable resources to replace petroleum as a primary feedstock for liquid fuels, chemicals and materials has become a topic of interest around the world. It is intriguing due to rising oil prices, the negative effects of petroleum on the environment and the advantages of renewable resources, such as their abundance and sustainability. Therefore, development of new technologies to broaden the product spectrum is necessary.

However, high value chemicals, including 1,4-butanediol (BDO), cannot be naturally produced by any known organism. Genetic engineering has emerged as a powerful tool for genetic manipulation of multistep catalytic systems involved in cell metabolism. Recombinant microorganisms, with altered sugar metabolism, are therefore able to ferment sugar to some specialty chemicals, which cannot be produced by the corresponding original strain. In fact, recent advances in process technologies, especially in fermentation technologies such as enzymatic engineering, metabolic engineering, and genetic manipulation, provide new opportunities for producing a wide variety of industrial products from renewable plant resources. To produce BDO by *E. coli* a biosynthetic pathway should be firstly constructed. The reconstructed strains design may serve as an important contribution to the implementation of bio refineries by converting biofuel waste - glycerol and, of course, glucose, into a higher-value chemical compound.

The main objective of the project is to produce bio based 1,4-butanediol (BDO) from the above-mentioned feedstocks using metabolically engineered *Escherichia coli* as the production strain growing on minimal medium (M9).

Content of the Thesis

The PhD Thesis is starting from the issues related to producing 1,4-butanediol (BDO) from renewable raw materials, including glucose or glycerin obtained from biodiesel production.

The doctoral thesis is structured on 6 chapters, developed on 90 pages, contains 15 tables, 53 figures and a bibliographic list with 111 titles, as follows:

Summary- presents the main objective, the hypotheses of the work and specific objectives for reaching the main object.

Chapter 1 – "***Literature overview"*** includes a presentation of the importance of 1,4 butanediol, the current market price, the chemical synthesis of BDO, the bio-based production of BDO, the importance of glycerol as a carbon source and methods for manipulating the target organism.

There is a significant increase in the production and use of bio-fuels, and especially the biodiesel and bio-ethanol production technology is growing year by year. The predictions is that glycerol produced annually will increase in the future and the development of different bio-based processes being able to convert low-priced glycerol into higher value products will be a big challenge and excellent opportunity. With this technology we will be able to add value to the production of biodiesel and make it much more competitive. The glycerol fermentation by different microorganisms could be a promising strategy to obtain a more important product.

Chapter 2 – The chapter "***Materials and methods***" include the original contributions of the author of the doctoral thesis, using the latest modern techniques for the genetic modification of the target microorganism.

To the best of our knowledge 1,4-butanediol is not synthesized by any known organism. Therefore, practically there are no biosynthetic pathways to increase the production of this unnatural compound. That is way the well-known *E. coli* was used as the host organism and the BDO biosynthetic pathway it was introduced into this organism. To reconstruct the metabolic pathways in this organism it is necessary to introduce enzymes isolated from other organisms. The introduction of heterologous enzymes was carried out by special plasmids. A potential strategy to improve BDO production was to block competing pathways, leaving only the BDO pathway to achieve redox balance during glucose or glycerol fermentation. After identifying the gene knockout strategies *in silico* using the bi-level optimization framework (OptKnock) the results was experimentally verified by using the λ-Red recombineering method to eliminate the competing pathways. The biggest challenges were the construction and expression of a

heterologous pathway to BDO from central metabolic intermediates, and the engineering of the carbon flux distribution. With these modifications the *E. coli* was able to produce BDO from lower-cost crude feedstocks at higher level, which will be economically more attractive. The production of BDO in genetically modified strain was optimized in bioreactor on minimal medium using different environmental conditions, culture conditions and carbon sources.

Chapter 3 – ***„Results***" is dedicated to the presentation of the performed experimental results, the metabolic flow analysis was performed in silico the results were verified experimentally and finally determined under the best conditions in the bioreactor, containing: the total genome analysis and the metabolic flow analysis in silico, the transcriptome analysis using DNA microarray, RT-qPCR, HPLC to measure expression levels of genes involved in different metabolic pathways and metabolite production.

Conclusions -. presents the general conclusions, the original contributions of the author and the main research directions in the future

APPENDIX –. contains additional information, protocols and image related to Chapter 2.

SUMMARY

The first hypothesis of this research is based on the possibility of using bioinformatics methods (capable of manipulating a huge amount of genomic, transcriptomic, proteomic and metabolomics data) to design and test new metabolic pathways which are naturally non-existent.

The second hypothesis of this work announced that by choosing a metabolite as a starting compound, using metabolic engineering (specific gene deletions of the competing pathways) and genetic engineering (expression and overexpression of heterologous genes) tools, the carbon flux can be directed by the newly introduced biochemical reactions to produce a non-natural molecule.

The main objective of this thesis was to design, develop and implement a new technology to produce 1,4-butanediol (BDO) with the genetic engineered *Escherichia coli* MG1655 which is able to use and convert renewable feedstock such as glucose and glycerol to added-value chemical compounds.

To achieve the target goal, the following steps have been completed:

- Modeling of bacterial metabolism and simulation of metabolic changes

To understand the cell as a system, it was necessary to use a complex metabolic model that contains information such as stoichiometry of metabolic reactions, chemical formulas, metabolite load, and relationships between genes, proteins and reactions. The most complex metabolic model of the *Escherichia coli* contains 1366 genes and more than 2500 enzyme reactions. While the 1,4-butanediol (BDO) is not synthesized by any known organism, the newly designed production pathway was not included in the base metabolic model (Biological Markup Language –SBML). After the insertion of this pathway into the Constraint Based Reconstruction and Analysis (COBRA) method of the MATLAB package, this updated program was used to predict the variety of metabolic phenotypes.

- Optimization of the production strain by metabolic engineering

A potential strategy for improving BDO production was blocking of the competing pathways, leaving only the BDO pathway to obtain the redox equilibrium. In the first phase, the identification of optimization strategies for the production of the target metabolite was performed *in silico*. According to that, the experiments were performed using the λ-Red recombination method. Since the main products are usually formate and ethanol during fermentation of glucose and glycerol, the following genes were needed to be deleted: *ldhA* (lactate dehydrogenase), *pflB* (pyruvate format-lyase), $adhE_2$ (alcohol/aldehyde dehydrogenase).

- Design of the modified metabolic pathway

In this study the most recent genome-scale metabolic model of *E. coli* was used and modified to be considered as the BDO biosynthetic pathway. The necessary reactions to facilitate the synthesis of BDO were identified by intensive literature mining; thereafter two non-natural reactions were identified. For the reconstruction of the new biosynthetic pathway, it was necessary to introduce heterologous genes encoding the enzymes required for the specific chosen reactions. The enzyme selection was based on specific databases, using the enzymatic properties (Km, Kcat/Km, substrate specificity, optimal temperature and pH, cofactors), protein properties (tendency to form inclusion bodies), and the phylogenetic distance of the organism as selection criteria. The enzymes found most active were analyzed, isolated and transformed into *E. coli* using specific expression plasmids capable of simultaneously expressing the required genes in the same cell. The transcriptional regulatory elements (T7 strong promoter) were used to ensure the expression of the enzymes in an appropriate amount.

- Experimental testing of the genetically modified strain

In order to determine the most important phenotypic traits of the obtained strains under various carbon sources, population dynamics and fitness testing was undertaken. The level of enzyme expression and the concentration of intermediate metabolites formed from the new biosynthetic pathway were also determined.

- Process optimization at laboratory scale

The production of 1,4-butanediol in genetically modified strains was optimized in bioreactor with minimal media (M9 media), using different culture conditions and different carbon sources.

CHAPTER I
LITERATURE OVERVIEW

The reserves of natural gas, oil and coal are increasingly becoming limited due to the continuous exploitation of fossil energy sources. In the long run, the biomass-based routes could be a good solution to produce bulk chemicals [1-4]. The petrochemical production processes generally are capital- and energy-intensive, produce chemical waste and pollution. One good alternative for sustaining growth of the chemical industry is to supplement with renewable resources [5]. It has been found that many biomass-derived chemicals have economic advantages, particularly for some functionalized chemicals. Therefore, there has been a growing interest in the utilization of biocatalysts to convert renewable resources into chemicals. Moreover, compared to chemical conversions, operating conditions for biological conversions are relatively mild and the processes take place with high yield and selectivity, and fewer byproducts. Biological conversions is considered to be the most flexible method for conversion of biomass into industrial products, involve the utilization of biological enzymes or living organisms to catalyze the conversion of biomass into specialty and commodity chemicals [6]. However, because of the metabolic restriction in microorganisms, only a few bulk products currently are produced via fermentation (such as ethanol, lactic acid, citric acid and acetone-butanol) [6-8]. Therefore, development of new technologies to broaden the product spectrum is necessary. In fact, recent advances in process technologies, especially in fermentation technologies such as enzymatic engineering, metabolic engineering and genetic manipulation, provide new opportunities for producing a wide variety of industrial products from renewable plant resources [9]. Genetic engineering has become an effective tool for genetic manipulation of multistep catalytic systems involved in cell metabolism widely used to create new high-performance cellular systems that convert abundant and inexpensive carbohydrates into bio-based fuels, chemicals

and polymers [3, 10]. In many cases the metabolic pathway of these chemicals is missing from the cell so the first step of developing biological production methods requires to build a biosynthetic pathway [11], achieving that the recombinant microorganisms, with altered sugar metabolism, will be able to ferment sugar to some specialty chemicals, which cannot be produced by the corresponding original [8].

The importance of 1,4-butanediol

1,4-butanediol is an important commodity chemical, an organic compound used as an intermediate to synthesize other chemicals and polymers [12].

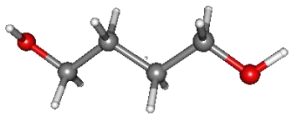

Fig.1.2.1. Chemical Structure Model of BDO [13]

Due to its bi-functional reactivity from the two OH groups (**Fig.1.2.1.**), it is one of the most important components in the manufacturing of several types of plastics including polybutylene terephthalate (PBT), elastic fibers (Spandex) [14], polyurethanes (car bumpers), a large family of homopolymers and copolymers [12, 15].

Methods and optimization steps for production strains

In order to create a bio-based production strain, several conditions need to be investigated. First and the most important is the selection of the heterologous host. The choice of the microorganism for metabolic engineering applications in most cases are determined by the following criteria: easiness of manipulation, cultivation conditions, the known of the genomic sequence and metabolic networks.[63, 64]. Model organisms like *Escherichia coli* and *Saccharomyces cerevisiae* fulfill these qualities and there are used on the development of many redesign strategies, to increase the productivity and yield of the target products [65, 66].

Another criterion that must be considered is the selection of enzymes. Enzymes are the basic elements of heterologous biosynthetic pathways and can be obtained from different sources. Series of databases, sequences and functional properties underlying a rational selection of enzymes that catalyze individual steps of newly inserted pathways [68-71]. Enzymes with

different biosynthetic backgrounds offer the possibility to combine them for converting to non-natural substrates and to perform a series of reactions for a defined purpose [68].

CHAPTER II
MATERIALS AND METHODS

This chapter describes the design of the new biosynthetic pathway that leads to 1,4-BDO production in details. In order to increase the concentration of the starting molecule (*succinil-coA*) of the new pathway, all other metabolic routes potentially competitive with the new heterologous route were bypassed by the deletion of key genes *(ΔldhA*, *ΔpflB* and *ΔaldhE$_2$)* using the λ-Red recombination method [51]. The formed succinic acid quantity was monitored by HPLC.

For our alternative heterologous pathway, the double specificity *aldehyde/alcohol dehydrogenase* from *Clostridium acetobutylicum* (*adhE$_2$* EC 1.1.1.1) and the native enzyme of *E. coli*, *succinyl-CoA synthetase* (*sucCD* EC 6.2.1.5) were selected. The vectors were checked by sequencing and their functionality were tested by transformation into T7 expression *E. coli* BL21(DE3) and into the metabolically optimized strains [95]. Expression of BDO-pathway enzymes under the control of T7 promoter was achieved after (DE3) lysogenization of the knocked-out strains. Methods of transformation including the preparation of cells for transformation, selection of transformants based on the antibiotic resistance gene located on the plasmid, propagation of colonies containing the target enzyme genes, induction of the system to overproduce the desired protein, isolation, purification and activity measurements of the protein are described in this Chapter II.

Knowing that the expression of recombinant proteins and gene deletions can significantly influence cellular viability, we investigated the impact of expression of the key enzymes by using deletion strains. This helped us to analyze the physiological changes of *E. coli* and provided directions for further optimizations our system to achieve satisfying target product yields.

After strain optimizations, culture conditions of both the optimized and the heterologous strains were analysed at microaerobic and anaerobic (oxygen-restricted) conditions, by using either glucose or glycerol as carbon source. The formed 1,4-BDO quantity were also monitored by HPLC.

In silico analysis of 1,4-BDO production

The genome-scale metabolic model of *E. coli*, iJO1366 [96], was used and modified to account for the BDO biosynthetic pathway. Heterologous reactions for 1,4-butanediol formation were selected based on literature sources and specific databases (KEGG; ECOCYC;

BRENDA; PDB; NCBI). Thereafter we analyzed the key enzymatic, structural and genetic parameters and properties like substrate specificity, k_{cat}, molecular weight, subunit composition, phylogenetic distance from the host, GC content etc. The reactions were identified and added into the original model considering the reactions stoichiometry. Minimal (M9) medium was selected for simulations, the uptake rate for the carbon source was set to 20 mmol $gDW^{-1}h^{-1}$, the oxygen uptake rates were set as follows: 0 mmol $gDW^{-1}h^{-1}$ for anaerobic, 5 mmol $gDW^{-1}h^{-1}$ for microaerobic and unbound for aerobic. The above values were chosen based on experimental observations reported in earlier studies [97-99]. In all simulations biomass calculations were carried out using MATLAB (The MathWorks Inc., Natick, MA,USA) and COBRA Toolbox software packages with TOMLAB CPLEX (Tomlab Optimization Inc., San Diego, CA, USA) [100, 101]. In order to optimize BDO production, we implemented OptKnock [102] and GDLS [103] into COBRA and analysed the effects of possible gene deletions in enhancement of the BDO flux. The gene knockout strategies were identified by using constraint-based modelling and the OptKnock and GDLS algorithms. The OptKnock strain-design methodology has been successfully used in a similar manner by Yim et al. to identify multiple sets of knockouts leading to the coupling of 1,4-butanediol production [12].

Competing pathway elimination

Chromosomal gene deletions were acomplished by using the λ-Red recombination method described by Datsenko and Wanner in 2000 [51].

Table 2.2.5.1. List of the created mutant strains

ELIMINATED GENES	RELEVANT GENOTYPE	EXPLANATION	SIGN
pyruvate format-lyase	MG1655 Δ*pflB*	*pflB* deletional mutant	MG14D1
pyruvate format-lyase, lactate dehydrogenase	MG1655 Δ*pflB*Δ*ldhA*	*pflB, ldhA* double deletional mutant	MG14D2
pyruvate format-lyase, lactate dehydrogenase, alcohol/aldehyde dehydrogenase	MG1655 Δ*pflB*Δ*ldhA*Δ*adhE*$_2$	*pflB, ldhA, adhE* triple deletional mutant	MG14D3

Investigation of the formed metabolites

Two-phase culture technique was used to study the succinic acid production of the triple mutant MG1655 Δ*pflB*Δ*ldhA*Δ*adhE*$_2$ bacteria compared to the control MG1655 strain. The first aerobic growth phase was followed by the anaerobic production phase. Cells were cultivated in 5 ml LB in an oxygenated environment shaking by 150 rpm at 37°C overnight, then harvested by centrifugation at 4500 rpm for 5 min, resuspended in fermentation medium and transferred

into a 100 ml flask containing 20 ml fermentation broth. Cultures were cultivated further at aerobic conditions shaking at 150 rpm at 37°C for 16 hour. Following this aerobic phase, cells were harvested by centrifugation at 4500 rpm for 10 min and resuspended in fresh fermentation broth. Cultures were transferred aseptically into sealed 150 ml bottles containing 75 ml of M9 medium supplemented with 5 g/L glycerol. The air in the headspace was replaced with oxygen free CO_2 gas, and bacteria were cultivated shaking by 150 rpm at 37 °C for 120 hours. To analyse extracellular metabolites, 1 ml of culture sample was harvested by centrifugation at 14000 rpm for 10 min, the supernatant was then filtered through 0.2 µm pore-size syringe filter for high-performance liquid chromatography (HPLC-Varian ProStar) analysis. Filtered samples were stored at -20 °C until use.

Vector construction for the new biosynthetic pathway

Since the designed biosynthetic pathway requires the parallel presence of two or more heterologous enzymes, we decided to use the pETDuet system capable for the simultaneous expression of two genes with a strong promoter developed by Novagen (purchased from Merck-Millipore).

In silico design and implementation

In silico design of the vectors was carried out by using the trial version of SnapGene. pETDuet1-*adhE2* co-expression vector (pGS1) DNA sequence encoding the double specificity *alcohol/aldehyde dehydrogenase* (*adhE2*) of *Clostridium acetobutylicum* was optimized for codon usage in *E. coli* and obtained by chemical synthesis (GeneScript, Hong Kong). For the construction of the pETDuet1-*adhE2-sucCD* (pGS1.1) for gene amplifications, the conventional PCR-directed molecular cloning methods were used with gene-specific primers using the genomic DNA of *E. coli* MG1655 (*sucCD*) strain as template.

Heterologous expression of the key enzymes

In order to investigate the target enzymes, we expressed the proteins by using *Escherichia coli* BL21 (DE3) strain. Chemically competent cells were transformed by 1 µL pGS1.1 as described in chapter 2.2.2. 200 µl of transformed cell suspension was spread on LB agar plates supplemented with ampicillin, and plates were incubated at 37°C, overnight. The chosen vector system offers the possibility to obtain proteins in high quantities (due to the strong promoter used), containing specific amino acid sequence (tags) through which the separation and purification of the proteins of interest can be achieved by affinity chromatography.

Protein purification

Purification of the alcohol/aldehyde dehydrogenase recombinant protein with a 6xHis was carried out by immobilized metal (Ni2+) affinity chromatography (IMAC). The histidines of the His-tags can chelate the metal, thus reversibly bind to the resins and separated from the un-tagged proteins and other components of the cell lysate. High concentration of imidazole containing buffer release the protein from the resin containing Ni2 +, this way isolation and concentration of His-tagged recombinant proteins can be achieved.

The introdution of the designed pathway into optimized strains

To ensure the optimal enzyme production of the new heterologous biosynthetic pathway, we utilized DE3-lysogenization (λDE3 Lysogenization Kit – Novagen, Merck Millipore) on the knocked-out strains. This system was designed for site-specific integration of λDE3 prophage into an *E. coli* host cell chromosome, such that the lysogenized host can be used to express target genes that are cloned in pET vectors under the control of T7 promoter. λDE3 is a recombinant phage carrying the cloned gene for T7 RNA polymerase under *lacUV5* control, thus expression of T7 RNA polymerase can be tightly regulated by IPTG induction in cells. Lysogens are prepared by co-infection with the three provided phages and tested with IPTG induction. To obtain production strains, chemically competent optimized mutant strains (MG14D1, MG14D2 and MG14D3) were transformed by heat-shock with co-expression vectors pGS1 and pGS1.1. Transformed colonies were selected on LB plates containing 50 μg/ml ampicillin.

Stability analysis of the BDO-producing strains

In order to evaluate the stability of plasmid maintenance and heterologous gene expression of the MG14D3-pGS1.1 strain, fermentation tests were maded under aerobic and anaerobic condition in M9 medium supplemented with 50 μg/mL ampicillin and 5g /L glucose. Induction of gene expression was performed at the time of inoculation by addition of IPTG at 0.5 mM final concentration, and cultures were maintained by shaking at 250 rpm, at 37°C.

Cultures obtained from the metabolically optimized (M14D3, from which the *pfl*B, *ldh*A and *adh*E genes were deleted) transformed (strains containing the pGS1.1 DNA vector) strain were made in volumes of 100 mL, in M9 medium, supplemented with ampicillin for maintaining plasmids during cultivation in anaerobic vessels (anaerobic conditions are ensured by purging the culture medium with CO_2 prior to flushing). Induction of biosynthetic pathway gene expression was performed with 1 mM IPTG (isopropyl-thiogalactopyranoside), added at the time of inoculation. During the cultivation, the growth of the number of cells was monitored

by measuring the OD_{600} values, and the concentrations of the metabolites of interest were determined in the intervals specified in the production culture supernatant according to the elaborated bioanalytical methodology.

Gene expression analysis

Gene expression analysis of BDO production strains, containing the biosynthetic pathway enzymes were performed by using microarray technics under culture conditions utilizing either glucose or glycerin as carbon source. Validation of the data were obtained by gene-specific RT-qPCR experiments. Total RNA preparations of production cultures (MG14D2-pGS1.1) grown under microaerobic conditions were extracted at the phase, when culture density reached the exponential growth (OD600 = 0.7-1), by using Thermo GeneJet RNA Purification Kit according to the protocol described by the manufacturer.

Transcriptome analysis with microarray

Determination of changes in gene expression could provide valuable information about the cellular metabolic network. Microarray technology can be used to characterize the gene expression profile of bacterial strains, when appointing of relevant changes at expression level can be correlated to clusters of genes with defined biological functions. The transcriptome analysis of the M14D2-pGS1.1 production strain compared with the M14D2-pC (harboring empty plasmid) reference strain grown under similar conditions were performed in the UD-Genomed laboratories in Debrecen (University of Debrecen, Hungary) using the GeneChip 3000 7G microarray platform with Autoloader and Scanner GCS 3000 7G, FS450 Fluidic Station and HO 645 Hibridization Oven respectively. Specific labeling of mRNA molecules appropriate for GeneChip E. coli array hybridization (Affymetrix) was performed by reverse transcription with random hexamer oligonucleotides for cDNA synthesis followed by DNaseI fragmentation and labeled with terminal transferase and biotinylated GeneChip DNA Labeling Reagent at the 3' termini. The initial total RNA used for this experiment was ~5μg. The fragmented cDNA was hybridized to the GeneChip E. coli array, using a minimal amount of 1 μg. The hybridization was performed in a HO 645 hybridization furnace, the array was flushed, tagged and scanned using a GCS 3000 7G scanner or the FS450 Fluidic Station automatic platform. Experimental data was analyzed (filtering and statistical analysis of relevant data) by using the GeneSpring software, which resulted a list of genes relevant to gene expression modification.

Microarray results validation by RT-qPCR

Validation of the data obtained from microarray transcriptome analysis were performed by using RT-qPCR, starting from the isolated total RNA samples obtained from the production cultures. The cDNA synthesis was performed by the RevertAid First Strand cDNA Synthesis Kit (Thermo) according to the the manufacturer intructions by using oligo(dT) primer for selective cDNA synthesis based on mRNA. The PCR reactions were started from 0.5 μg of synthesized cDNA using Mx3005P (Agilent) platform. For labeling the Maxima SYBR Green qPCR Master Mix fluorescent label (Thermo Scientific) and for reference the fluorochrome ROX were used. Reactions were performed in triplicates for a set of three genes of interest from the point of view of the introduced biosynthetic pathway (*pflB, sucCD, pykF*) using the 16SrADN region as internal control gene.

Small-Scale Aerobic/Anaerobic Growth

Starter cultures were prepared by inoculating individual colonies in 5 mL of LB broth and incubated shaking at 160 rpm at 37°C overnight. Cells were centrifuged by 5000 rpm for 10 minutes, pelleted cells washed with PBS and resuspended with 1 mL of culture broth then transferred into a sterile 100 mL Erlenmeyer flasks containing 25 ml M9 broth. In the case of microaerobic/anaerobic conditions, the starter cultures were prepared the same way, then bacteria were placed into 150 mL sterile anaerobic flasks containing 100 mL medium. In case of anaerobic cultures, the media was flushed with CO_2 for 1 minute through a 0.45 μm filter (Millipore) before inoculation. All versions of cultures were prepared in triplicates. The production cultures (e.g. cultures of mutant strains harboring pGS plasmids) were induced with 1 mM IPTG (Sigma). Optical density was followed with a CamSpec M330 spectrophotometer at 600 nm.

Fermentation Set-Up

Inoculum bacterial cultures were prepared by transferring 200 μL of frozen glycerol stock of strain in 5 mL of LB broth and incubated shaking at 150 rpm at 37°C for 4 hours. Starter cultures were obtained by dilution of 2 mL inoculum culture in 100 mL M9 mineral medium and further cultivated by agitation in a rotary shaker at 150 rpm at 37°C for 16 hours. The appropriate volumes of bacterial culture corresponding to desired starting OD_{600} values were harvested by centrifugation at 5000 rpm for 10 minutes, cell pellet washed with PBS, resuspended in culture broth and cells were then added into the reactor vessel containing 1 L of media. Fermentations were carried out in a 2-L Sartorius Biostat®A Plus Bioreactor, using a

BioPAT® MFCS/DA data acquisition and control system. Temperature was set to 37°C, pH to 7.0. This pH was maintained using NaOH and HCl throughout the fermentation. The agitation was set to 1300-rpm in case of aerobic cultures, airflow was continuous along the fermentation process, set to 1 VVM (vessel volume per minute), whereas the dissolved oxygen (DO) was set to 40%. For microaerobic conditions, the dissolved oxygen (DO) was set to 5%, sparging the reactor headspace with a flowrate of 0.05 l/min.

Analytical Methods

Metabolite analyses were carried out by Agilent Infinity 1260 chromatography system equipped with UV-VIS and RID detectors and auto-sampler. Isocratic determination of glucose, BDO, succinate and acetate were carried out using a Coregel 87H3 column maintained at 50°C, with 0.008 N H_2SO_4 as mobile phase, at 0.6 ml/min flow-rate. Samples were prepared by centrifugation of cultures at 10000 rpm for 10 minutes, supernatant was filtered through 0.2 μm Millipore filters and stored, when needed, at -80°C. Calibration was carried out with HPLC grade pure compounds purchased all from Sigma.

CHAPTER III
RESULTS

Pathway design

The new biosynthetic pathway employs the 4-carbon TCA cycle intermediate *succinyl-CoA*, which, in three enzymatic reduction/CoA activation steps is converted into 1,4-butanediol (**Fig.3.1.1.**). The designed biochemical pathway leading to BDO production is starting from *succinyl-CoA*, which can be converted into 4-hydroxybutyrate by the double specificity aldehyde/alcohol dehydrogenase from *Clostridium acetobutylicum* (*adhE2*). The carboxylic group of 4-hydroxybutyrate can be activated by the native enzyme of *E. coli*, *succinyl-CoA synthetase* (*sucCD*) and in the final step the 4-hidroxybutyryl-CoA will be converted to 1,4-butanediol by the same *adhE2*.

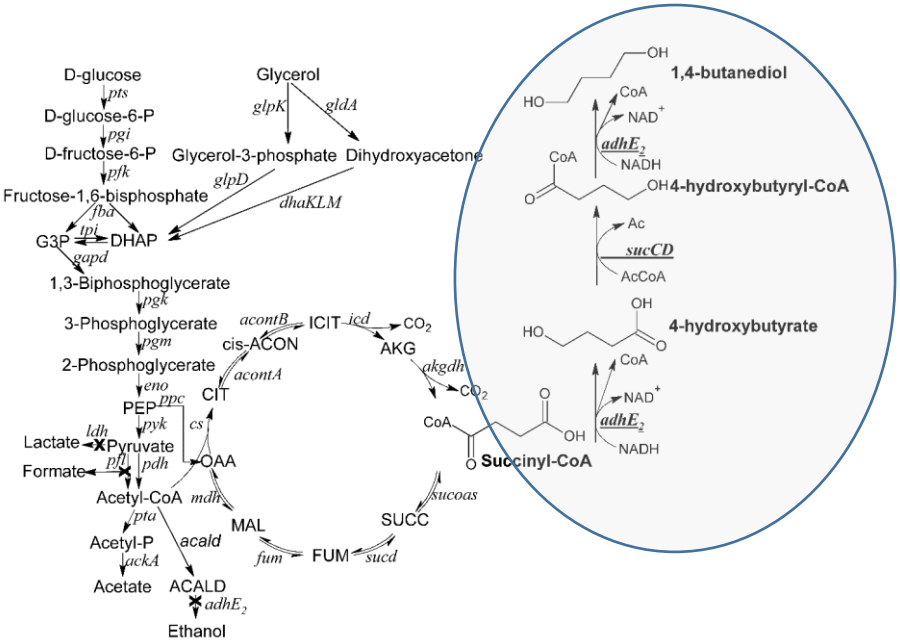

Fig. 3.1.1. Proposed biosynthesis pathway for 1,4-butanediol formation

The proposed pathway is shown highlighted, the eliminated reactions are represented by a black "X".

For optimizing the yield of the target product blocking off the competing metabolic pathways is one of the promising strategies. With *ldhA* gene deletion the lactic acid production, with deletion of the *pflB* gene the formic acid production and with knocking-out the *adhE*$_2$ gene the ethanol production could be inhibited, redirecting the carbon flux to increase the formation of *succinyl-coA*, which is the starting molecule of our newly designed route.

Flux balance analysis was used for phenotype analyses to estimate the flux distribution and determine if the pathway is active under certain conditions. It is a linear optimization method (LP) based on stoichiometric knowledge of the organism's metabolic network. Simulations were carried under steady-state conditions:

$$\max Z = c^T v \quad (1)$$

$$\text{subject to } S\,v = 0 \quad (2)$$

$$\left(\sum_j S_{ij}\, v_j = 0\right) \quad (3)$$

$$v_{lb} < v < v_{ub} \quad (4)$$

where:

- Z -is the objective function,
- c-is a vector of weights (the contribution of each reaction to the objective function),
- S-is the stoichiometric matrix with m metabolites and n reactions,
- v flux vector with n elements,
- v_{lb} and v_{ub} represent the lower and upper limits on the fluxes, respectively.

Considering the predictions, the BDO pathway is inactive in the wild-type *E. coli* MG1655 strain even if the oxygen consumption is reduced/eliminated or the carbon source is switched from glucose to glycerol. Bi-level optimization was carried out to find gene/reaction candidates leading to BDO formation while maintaining optimal growth (growth at least 0.1 h^{-1}). Results with highest BDO productions are presented in **Table 3.1.1.**

Table 3.1.1. In silico obtained modelling results, compared with other works [107]

SUBSTRATE	BIOMASS FLUX (H^{-1})	BDO (MMOL $GDW^{-1}H^{-1}$)	BY-PRODUCTS (MMOL $GDW^{-1}H^{-1}$)		CONDITIONS	REFERENCE
GLUCOSE	0.30	14.46	Acetate	21.06	Anaerobic	Own results
	0.14	15.17	Acetate Pyruvate Ethanol	- - -	Anaerobic	[12]
	0.39	10.27	-	-	Anaerobic	[108]
GLYCEROL	0.25	8.68	Acetate	7.49	Microaerobic	Own results

Optimal fermentative conditions predicted for glucose could be without oxygen (anaerobic) with 3 KO (*ΔpflB, ΔldhA, ΔadhE*$_2$,) mutations, where the molar yield was over 0.7 mol BDO per mol glucose. In case of glycerol only microaerobic conditions seem to be adequate for BDO production.

Obtaining gene-specific linear cassettes

In order to produce the mutant strain gene-specific cassettes were prepared by polymerase chain reaction, amplifying the chloramphenicol/kanamycin resistance gene, having also chain-linked ends with the target region of *E. coli*. The success of the PCR reaction was verified by 1% agarose gel electrophoresis.

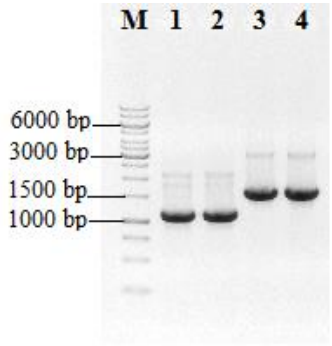

Figure 3.2.1. Verification of specific gene cassettes in agarose gel 1%

M: 1 kb DNA marker (Fermentas), 1µl; 2, 2, 3: gene-specific cassettes harboring chloramphenicol (1100 bp) resistance; 4,5: gene-specific cassettes harboring kanamycin (1600 bp) resistance

On the figure above we can observe that the bands corresponding to the expected sizes: 1100 bp for chloramphenicol resistance and 1600 bp for kanamycin resistance cassettes.

Transformation with pKD46

Once we obtained the linear gene-specific cassette we created chemically competent E. coli cells. The express of the Red recombinase genes are required for the integration of the cassette so the cells were transformed with pKD46 plasmid.

Verification of the gene elimination by PCR

The fact of the gene replace with the proper cassette was verified with colony PCR, The PCR product was checked on a 1% agarose gel, the result being illustrated in Figure 3.2.4.1.

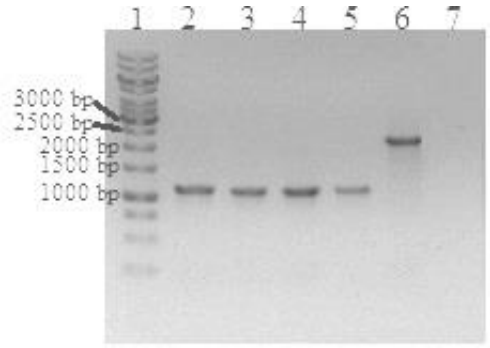

Figure 3.2.4.1. Verification of *pflB* gene exchange with the gene-specific cassette
Column 1. 1 kb DNA marker (Fermentas), Column 2-5: gene-specific PCR products, Column 6: *pflB* gene of E.coli MG1655, Column 7: no template control

According to the gel, we can confirm that replacing of the *pflB* gene with the chloramphenicol cassette was successful (corresponding to the size of the cassette 1100 bp). The DNA amplicons appeared in column 6 is identical to the size of the genomic *pflB* gene of the *E. coli* MG1655, having a size of 2295 bases pairs.

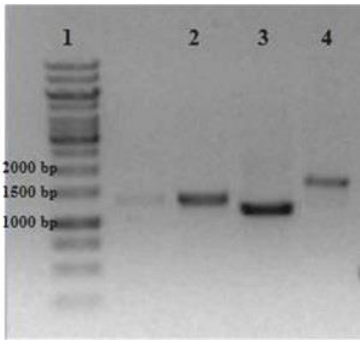

Figure 3.2.4.2. Verification of *ldhA* gene exchange with the gene-specific cassette
Column 1. 1 kb DNA marker (Fermentas), Column 2: *ldhA* gene of *E.coli* MG1655gene-specific PCR products, Column 3: gene-specific PCR products obtained for MG14D1, Column 4: gene-specific PCR products for Δ*pflB* Δ*ldhA*

The DNA amplicons appeared in column 2 is identical to the size of the genomic *ldhA* gene of the *E. coli* MG1655, having a size of 1319 bases pairs. The replacing of the *ldhA* gene with the chloramphenicol cassette was successful (corresponding to the size of the cassette 1600 bp).

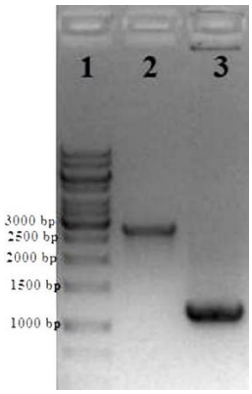

Figure 3.2.4.3. Verification of *adhE₂* gene exchange with the gene-specific cassette
Column 1. 1 kb DNA marker (Fermentas), Column 2: *adhE₂* of E.coli MG1655, Colum 3: gene-specific PCR products for Δ*pflB* Δ*ldhA*Δ *adhE₂*

According to the given result we can confirm that exchange of *adhE₂ gene* was also successful, the gene-specific PCR product was 1100 bp, being evidence for the presence of the chloramphenicol cassette.

Antibiotic resistance elimination

In order to remove the antibiotic resistance gene, pCP20 plasmid was introduced into the cells by electroporation followed by selection of cells containing the plasmid on ampicillin-containing media. The selected colonies were inoculated into a new plate and incubated at 43°C for 2 days.The mutation were also verified with PCR reaction. The size of the PCR product corresponds to the size of the two FRT regions of the cassette (200 bp).

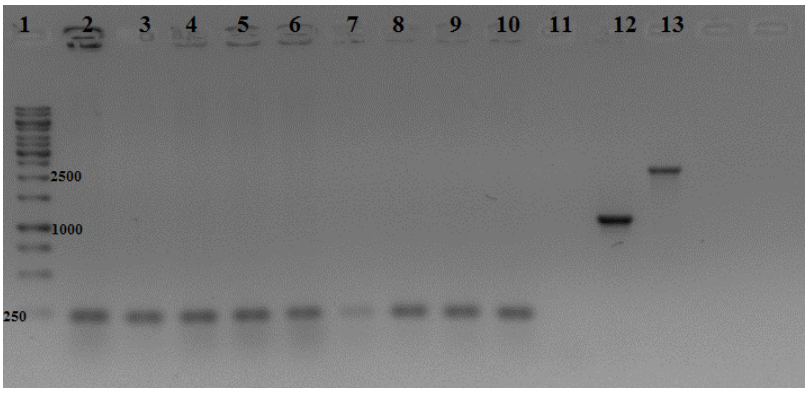

Figure 3.2.5.4. Electrophoretic migration of PCR products of *E. coli* MG1655 Δ*pflB* mutant strains 1% in agarose gel
Column 1: 1 kb DNA marker, Fermentas; Column 2-10: PCR product given with verification pflB3 and pflB4 primers, Column 11: No template control, Column 12: linear gene-specific cassette for elimination of *pflB* gene; Column 13: *pflB* gene of *E.coli* MG1655

As we can see were the gene-specific cassettes disappeared, at 1100 bp we couldn't observe any bands.

The analysis of the produced metabolites

The concentration of metabolites in culture broth were determined by HPLC (Varian ProStar), the system was equipped with refractive index detector, UV detector and an ion-exchange column (7.8 mm x 300, ICSep Coregel 87H3, Transgenomic). The mobile phase was 0.008 N H_2SO_4 and the flow rate 0.6 ml/min during elution. The column temperature was 50°C. Succinic acid were measured by the UV detector at 210 nm and sugars were measured by the RI detector.

The obtained chromatograms are showned belove.

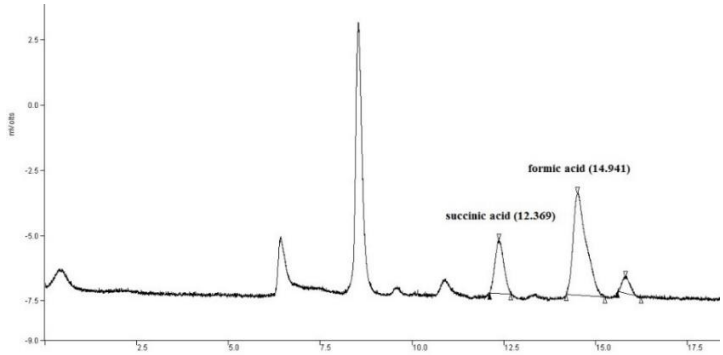

Figure 3.2.6.1. Metabolites produced by *Escherichia coli* MG1655after 120 hour of fermentation

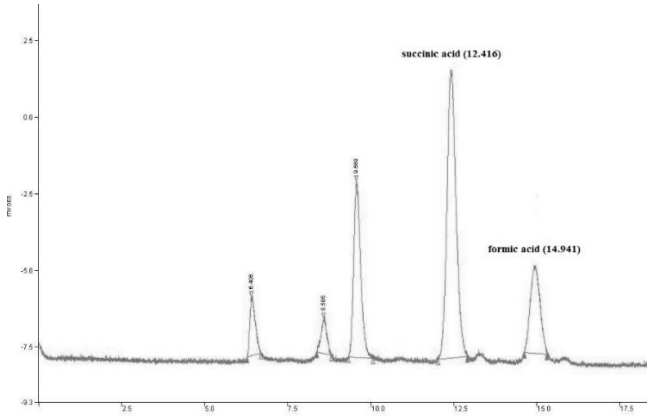

Figure 3.2.6.2. Metabolites produced by *Escherichia coli* MG1655 Δ*pflB*Δ*ldhA*Δ*adhE*$_2$ after 120 hour of fermentation

The retention time of the succinic acid is at 12.4 min. As we can see, by eliminating the proper genes, the production of the succinic acid significantly increase.

Heterologous protein expression and purification

Chemical transformation of the designed vector into chemically competent cells by heat shock was carried out as described in Materials and methods. Result of the transformation of pETDuet-1-*adhe-sucCD* (pGS1.1) plasmid DNA into *E. coli* BL21(DE3). The selection based on amp^r of transformed bacterial colonies. To produce the target protein the expression was induced by isopropyl β-D-1- thiogalactopyranoside (IPTG) in a final concentration of 0.5 mM. The culture was maintained at 37^{o}C in a shaking incubator at 250 rpm for 3h. In every phase of the experiment samples were collect and prepared. The recombinant *adhE$_2$* protein was purified by affinity chromatography. The success of the expression and purification were visualized with polyacrylamide gel electrophoresis (SDS-PAGE).

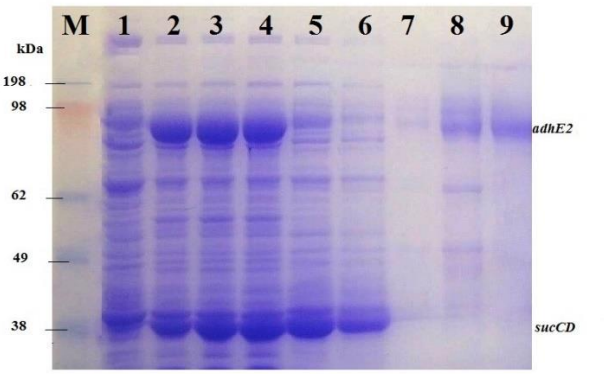

Fig.3.4.2. Heterologous proteins expressed from pGS1.1 and purification of *adhE2*

Cellular proteins derived from E. coli BL21 (DE3) transformed with the co-expression vector pGS1.1; the *adhE2* protein were purified by specific binding to Ni2 + -Sepharose. Samples were visualized with ComassieBlueR250.
M: Molecular weight marker SeeBlue Plus2 (Invitrogen); 1. cell culture supernatant of BL21(DE3)-pGS1.1 before induction; 2: cell culture supernatant after 1 hour of iduction; 3: cell culture supernatant after 2 hour of iduction,; 4: cell culture supernatant after 3 hour of iduction; 5: soluble proteins obtained from crude cell extract; 6: unbounded protiens to the Ni2+-Sepharose sresin (flowtrough);7-8: fracții proteice pure după eluție cu 300 mM imidazol; 9: dialised pure adhE$_2$

According to the results, the *E. coli BL21*(DE3) strain are suitable for the production of proteins from recombinant vector pGS1.1. After 1, 2 and 3 hours of induction a high amount of *adhE2* (94.6 kDa) formed compared to the controll (before induction). The overexpression of the native enzyme s*ucCD* (subunits 38 and 40 kDa) also were successful, the bands appeared on the size similar to the theoretical molecular weights. The purfication of the His-taged *adhE2* was also successfull and after elution and dyalisis we earned about 3 mg/mL pure protein.

Enzyme activity determination

During the determination of the enzyme activity of the pure *adhE₂* general and specific substrates were used. The *adhE₂* use NAD^+ or $NADP^+$ as coenzyme, which is reduced during the dehydrogenation. The reduced NADH or NADPH exhibits strong UV absorption at 340 nm. The reaction may be followed by measuring the increase in absorbance of the solution at 340 nm as NADH is formed. The substrate specificity was investigated using acetic acid, succinyl-coA and butyryl-coA which are the intermediate compounds of 1,4-butanediol production pathway. Based on the kinetic curves obtained the enzyme activity were calculated using the Beer-Lambert Law. The recorded kinetic curve and calculation method for determining the relative enzymatic activity are shown below:

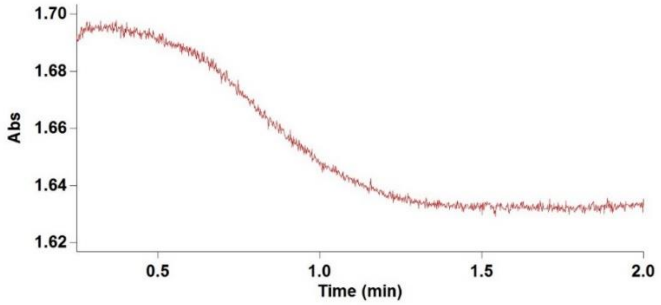

Fig.3.4.1.1. The activity of *adhE₂* on succinyl-CoA substrate

$\Delta_{Abs}/min = 0{,}038$

$$A = \varepsilon * c * L$$

$$c = \frac{A}{\varepsilon * L}$$

$$c_{product} = \frac{0{,}038}{6{,}22 * 10^3 * 1} = 0{,}0000061093 = 6{,}109\ \mu mol$$

$$U_{rel} = \frac{c_{product}}{m_{enzyme} * t} = \frac{6{,}109\ \mu mol}{12{,}7\ \mu g\ enzyme * min * mL}$$

$$U_{rel} = \frac{0{,}481\ \mu mol}{\mu g\ enzyme\ * min * mL} = 0{,}481\ mol\ \frac{substrate}{mg\ enzyme * min * L}$$

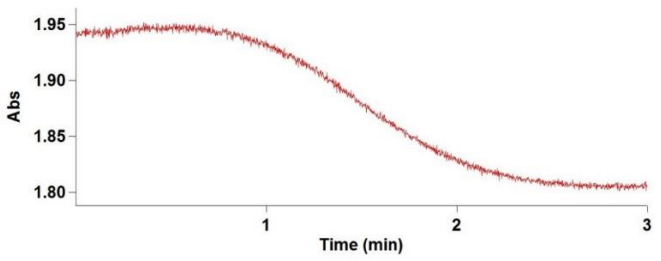

Fig.3.4.1.1. The activity of *adhE₂* on butyryl-coA substrate

$\Delta_{Abs}/min = 0{,}051$

$$c_{product} = \frac{0{,}051}{6{,}22 * 10^3 * 1} = 0{,}000008199 = 8{,}199\ \mu mol$$

$$U_{rel} = \frac{c_{product}}{m_{enzyme} * t} = \frac{8{,}199\ \mu mol}{6{,}351\ \mu g\ enzyme * min * mL}$$

$$U_{rel} = \frac{1{,}291\ \mu mol}{\mu g\ enzyme\ * min * mL} = 1{,}291\ mol\ \frac{substrate}{mg\ enzyme * min * L}$$

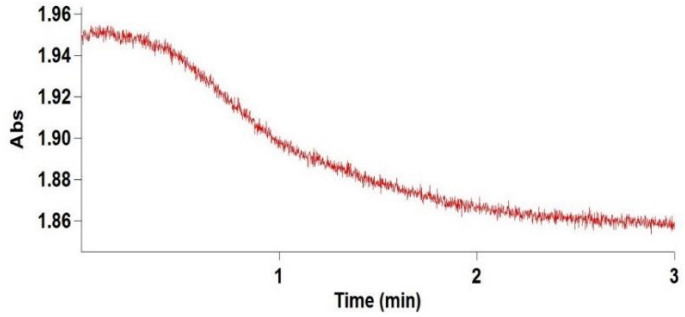

Fig.3.4.1.3. The activity of *adhE₂* on acetic acid

Δ_{Abs}/min = 0,033

$$c_{product} = \frac{0{,}033}{6{,}22 * 10^3 * 1} = 0{,}000005305 = 5{,}305\ \mu mol$$

$$U_{rel} = \frac{c_{product}}{m_{enzyme} * t} = \frac{5{,}305\ \mu mol}{3{,}177\ \mu g\ enzyme * min * mL}$$

$$U_{rel} = \frac{1{,}669\ \mu mol}{\mu g\ enzyme\ * min * mL} = 1{,}669\ mol\ \frac{substrate}{mg\ enzyme\ * min * L}$$

The results of the enzymatic activities for different substrates are summarized in Table 3.4.1.

Tabel 3.4.1. Values of enzymatic activities

ACETIC ACID	SUCCINYL-COA	BUTYRYL-COA
U $= \mathbf{0{,}481}\ \frac{\mathbf{mol\ subst}}{\mathbf{mg\ enzyme * min * L}}$	U $= 1{,}669\ \frac{mol\ subst}{mg\ enzyme * min * L}$	$U = 1{,}891\ \frac{mol\ subst}{mg\ enzyme * min * L}$

Gene expression analysis with Microarray

As a result of the complete analysis of the transcriptome of the developed strains, a list was obtained with the genes showing significant changes in gene expression level, the statistical analysis of the data being performed by defining the fold-change parameter. Relevant genes from the set of samples analyzed were compared from the point of view of the substrate used (glycerin: samples 1.2 and 2.2 or glucose: samples 1.1 and 2.1), or based on the activity of the

heterologous pathway (samples with control plasmid 1.1 and 1.2, respectively, samples with functional biosynthetic pathway 2.1 and 2.2). Thus, without identifying all of the genes with significant differences in fold-change terms, a number of 40 relevant genes from the level of expression was achieved, and they were grouped according to biological function and relevance at the point of view of the purpose of this stage: identification of intervention points in the metabolic pathways or in the control elements for increasing the productivity of the metabolically optimized strains. Since our main goal was to create a metabolic optimization in order to form the product coupled with the bacterial growth rate, especially when used as a glycerin substrate (a substrate that is more metabolized as glucose), in the transcriptomic analysis we focused on genes that can influence these two criteria. Relevant genes by biological function resulted in the identification of a cluster of genes related to glycerol metabolism, glpK (glycerol kinase), glpT (glycerol-3-phosphate transporter) glpF (glycerol transporter). Also glpQ (glycerophosphoryl-diester) periplasmic phosphodiesterase), showing increased gene expression of 4.09x, 3.26x, 2.22x, and 2.45x, respectively, expressed in fold-change in the case of the MG14D2-pGS1.1 producing strain (sample 2.2) compared to the reference strain (sample 1.2) in the presence of glycerol as a substrate. Surprisingly, glpC (glycerol-3-phosphate anaerobic dehydrogenase) has a lower expression level in the 1,4-BDO-producing strain (**Fig.3.6.1.1.**).

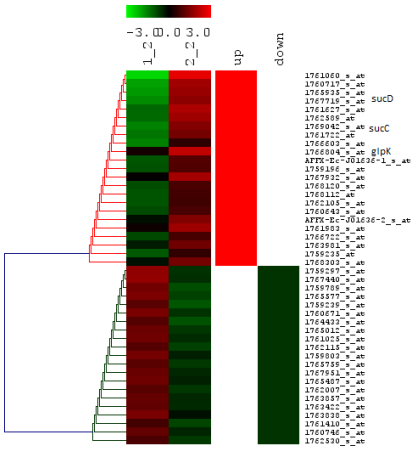

Fig.3.6.1.1 Relevant genes in terms of expression level change when comparing MG14D2-pGS1.1 production strain (sample 2.2) and reference strain (sample 1.2), raised on glycerol as the sole carbon source. (MeV 4.9.0)

Another group of identified genes are involved in the metabolism of pyruvic acid, respectively in the tricarboxylic cycle, these metabolic pathways being of major importance from the project point of view, as the intermediate metabolite from which the 1,4-BDO conversion is carried out

is succinyl-CoA, and competitive pathways, inhibited by the deletion of the ldhA and pflB genes, are reactions of pyruvic acid metabolism. As expected, the experimental data suggest an increased expression level of sucC and sucD (genes encoding succinyl-CoA synthetase subunits, one of the enzymes of the biosynthetic pathway, introduced by plasmid pGS1.1) of 8.47x, respectively 9.84x, or expressed in fold-change in the case of cultures grown on glycerin, and in the case of glucose utilization, as substrate, an even more pronounced level of expression is recorded, of 14.84x in the case of sucC and 12.52x in the case of sucD. Thus, it can be concluded that the recombinant plasmid carrying the biosynthetic pathway enzymes for 1,4-BDO is functional on the substrates used, providing an adequate level of expression to the strong T7 promoter, and can ensure the addition of an additional pool of succinyl-CoA. There was also a more modest increase in the genes related to the α-ketoglutarate dehydrogenase complex (sucA and sucB), with an increase in the expression level in the production strain of 1.64x (sucA) and 1.72 (sucB) in the case of glycerin and 1.74x (sucA) and 1.46 (sucB) in the case of glucose. The increased expression level of acnB (aconate hydrase), with 1.31x on glycerin and 2.12x on glucose in the 1,4-BDO-producing strain, suggests the functioning of the oxidative branch of the tricarboxylic cycle, and aceB (malate-synthetase) also has a level. increased expression (2.06x for glycerin and 1.36 for glucose). According to the experimental data, altered gene expression levels were observed for several genes with regulatory role, respectively maintaining cellular redox balance. Thus, there was a significant decrease in the expression of the rseC gene (redoxsensitive transcriptional activator) (Koo et al., 2003) in the producing strain as compared to the reference strain (3.94x in the case of glycerin and 3.93x in the case of glucose). a possible explanation could be the balancing of cellular redox conditions by the metabolic-genetic changes introduced into the producing strain. Another gene that plays a role in maintaining redox balance is nuoG (NADH-quinone oxidoreductase subunit G), which has an increased expression level of 2.27x in the case the production strain 2.2 on glycerin, respectively 1.45x on glucose.

Gene expression analysis with RT-qPCR

For the two-step RT-Q-PCR reactions, a set of 3 genes of interest were used in terms of the functionality of the introduced biosynthetic pathway (pflB, sucCD, pykF) using gene control, 16SrADN region. The amplification curves performed suggest a good correlation with the data obtained from the transcriptome analysis, the CT (cycle treshold) values in the case of sucCD gene being 23.56 in the production strain, and 18.87 in the case of sample 1.2 (**Fig.3.6.2.1**). In contrast, in the case of pyruvate kinase (pyk) no changes in expression level were observed

under the conditions of applied cultivation (**Fig.3.6.2.2**), registering CT values of 19.45 for 1.2, respectively 19.58 for sample 2.2.

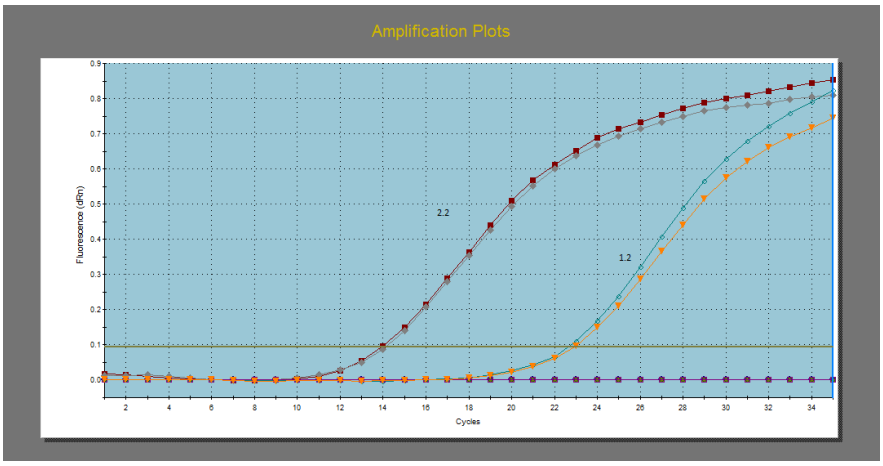

Fig.3.6.2.1. Amplification curve for sucCD-specific RT-Q-PCR reactions for the production strain (2.2) and the reference strain (1.2), grown using glycerin substrate

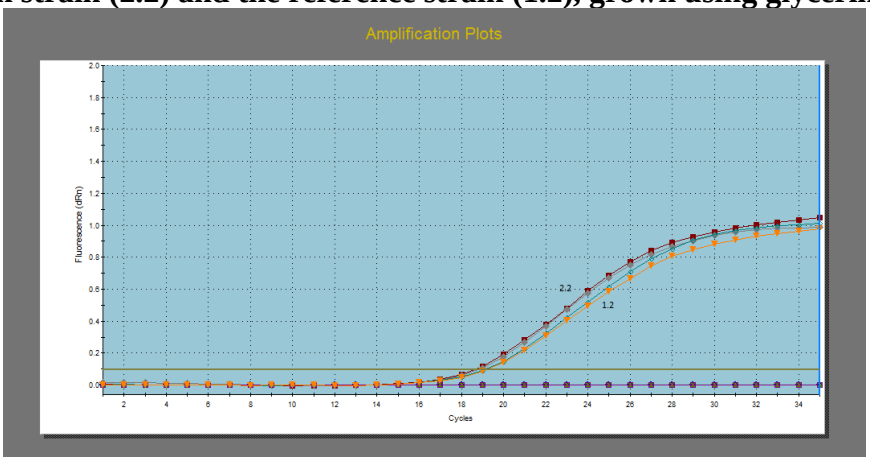

Fig.3.6.2.2. Amplification curve for pyk-specific RT-Q-PCR reactions in the case of production strain (2.2) and reference strain (1.2), grown using glycerin substrate.

3.1.Determination of Key Phenotypic Traits

3.1.1. Metabolic profiles of mutant strains

In order to determine the most important phenotypic traits of the obtained strains, small-scale anaerobic cultures were set up in triplicates and cultured as described in the previous section. Plasmid-harbouring cultures were induced with 1 mM IPTG at inoculation and ampicillin was added where appropriate. Culture growth was monitored based on OD_{600} values, and samples at indicated time-points were analysed by HPLC as described in the Experimental section. Results shown in **Fig.3.8.1.1.** depict the evolution of substrate, product and selected metabolite concentration during fermentations.

Acetate and succinate quantities in the culture supernatants of MG14D3, MG14D3-pGS1 and

MG14D3-pGS1.1 suggest that succinate and acetate were formed in the cultures, consistent with culture conditions, higher concentrations of the two metabolites varying with changes in substrate concentration (**Fig.3.8.1.1.B**).

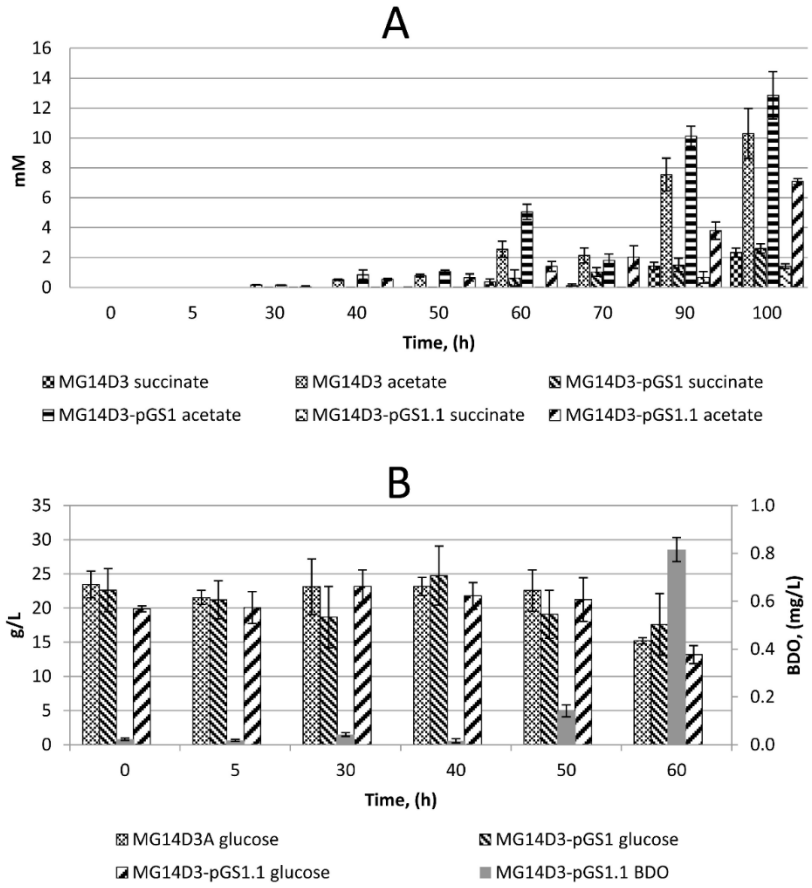

Fig.3.8.1.1.. Evolution of substrate, product and selected metabolite concentrations during fermentation.

A. Metabolic products of MG14D3, MG14D3-pGS1 and MG14D3-pGS1.1 anaerobic cultures.
B. Substrate and BDO concentrations in MG14D3, MG14D3-pGS1 and MG14D3-pGS1.1 anaerobic cultures.

Evaluation of optimal culture environment

After preliminary experiments, we studied stability of WT and BDO-producing strains under different fermentation conditions, starting with a few tests to assess the selected media composition (M9+NaCl/M6) efficiency compared to a complex medium in a bioreactor. Biomass production was followed firstly in case of the original strain (**Fig. 3.8.2.1.A**) in LB medium, under aerobic conditions and dry cell weight was determined in different time points. Our results suggest a good correlation between OD values and the determined DW values, with a maximal OD reaching 10.79 after 9 hours of culture.

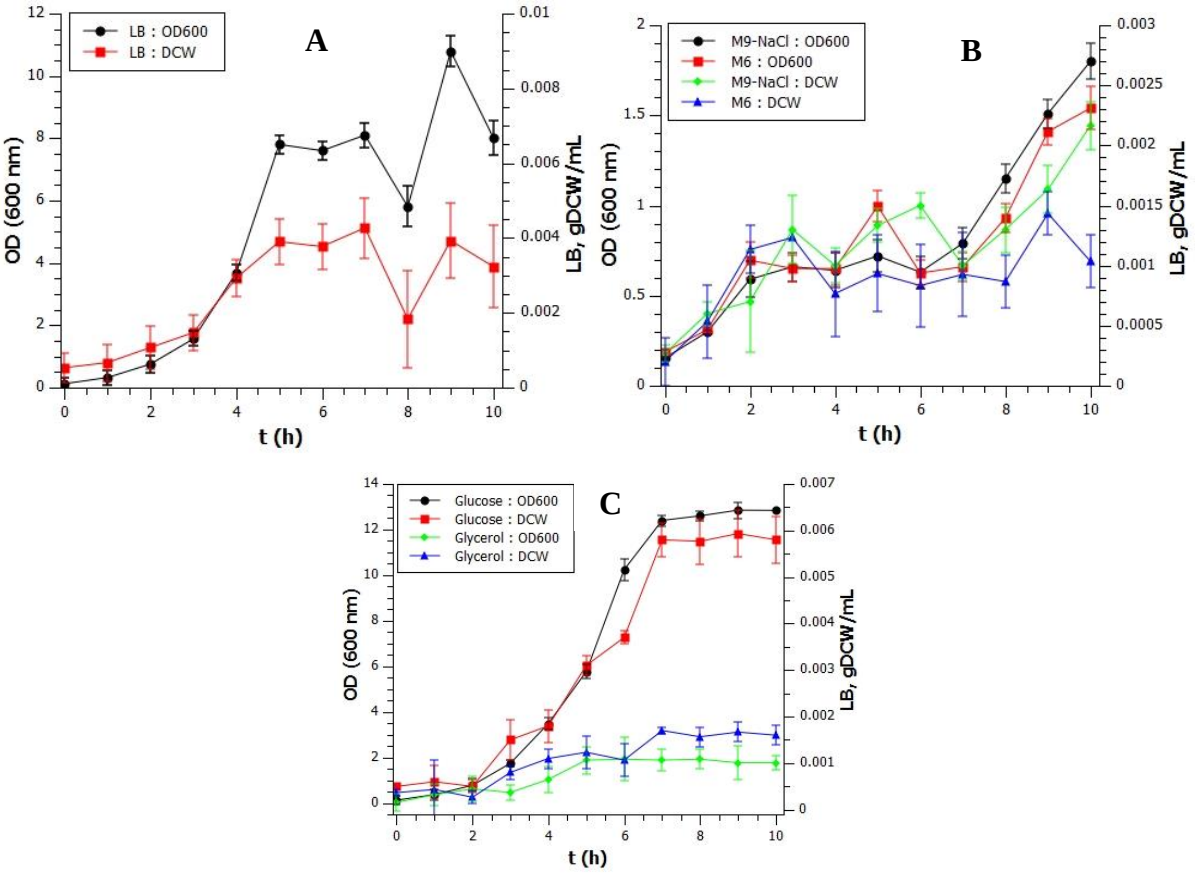

Fig.3.8.2.1 Growth of bioreactor cultures of MG1655 and MG14D3-pGS1.1 under aerobic and microaerobic conditions.

A. MG1655 cultured aerobically on LB medium, monitoring OD values and the corresponding DW values over the time course of cultivation.

B. Culture of MG1655 in a bioreactor, on glycerol, using M9+NaCl or M6. Values of OD600 and DW are represented.

C. Microaerobic bioreactor culture of MG14D3-pGS1.1, on M9+NaCl medium, with glucose/glycerol as the sole carbon source. Evolution of OD values vs. gDW/mL of culture is represented.

A series of experiments were performed to assess the effect of previously selected minimal media on the WT MG1655 strain using glycerol as the sole carbon source under aerobic conditions. Bioreactor experiments proved no significant influence of the two selected media (M9+NaCl/M6), M9+NaCl however, producing a slightly more biomass yield.

Microaerobic reactor set-up for BDO producing strain MG14D3-pGS1.1 was carried out in order to assess the effect of carbon source on bioreactor cultures of this strain. As a culture medium, we used the M9+NaCl mineral medium previously selected for the wild-type strain, with glycerol (5 g/l) or glucose (5 g/l) added in batch mode, DO being set at 25%. As depicted in **Fig. 3.8.2.1. C**, we could achieve an OD value of over 12 in case of glucose as a sole source of carbon, while for glycerol, we registered a maximal value of OD of 1.92, due to the poor utilization ability for this substrate [107].

3.2.Metabolic profile of MG14D3-pGS1.1 strain

After establishment of adequate culture conditions and evaluation of strain behaviour under different circumstances, bioreactor experiments were performed in order to evaluate changes in cellular metabolism in a very specific situation: microaerobic/anaerobic transition. To this scope, we set up bioreactor cultures of MG14D3-pGS1.1, in M9+NaCl medium with glucose and Amp, at 37^{0}C, pH 7 and 350 rpm initial agitation, controlling the dissolved oxygen quantity inside the medium by a constant flow of air in the reactor airspace. We monitored changes in pO_2, OD_{600}, substrate, product concentrations, as well as selected metabolite concentrations in the culture broth at certain time-points.

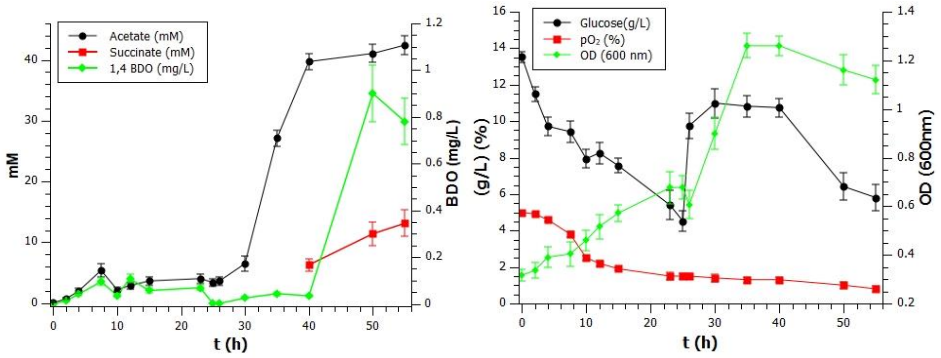

Fig.3.9.1.. Microaerobic/anaerobic transition in growth of MG14D3-pGS1.1 in bioreactor cultures.

A. Acetate, succinate and BDO production in MG14D3-pGS1.1 in bioreactor cultures.
B. Glucose concentrations, DO values and OD values of MG14D3-pGS1.1 in bioreactor cultures.

As shown in **Fig.3.9.1.** cells started to produce acetate, succinate and BDO at the border of anaerobiosis (pO_2 value around 1%), however, we could find detectable levels of BDO (0.89 mg/l) in samples corresponding to 50 hours of culture [107].

CONCLUSIONS

Pathway engineering and host optimization may cause metabolic imbalances and induce secretion of secondary metabolites (Hollinshead et al., 2014). Moreover, genetic modifications often increase the metabolic burden on the host, e.g. by using high copy number plasmids or strong promoters, which can, in turn affect cell growth, and consequently, overall productivity. Metabolic flux analysis has revealed that the overexpression of biosynthesis pathway genes significantly increases ATP maintenance expenditure (Ow, Lee, Yap, & Oh, 2009). Therefore, modern fermentation engineering should focus on reducing the metabolic burden and enhancing

the prosperity of the energy metabolism. Several effective strategies can be applied to improve NAD(P)H and ATP availability, such as: adding nutrient sources (such as yeast extract and other additives) to culture media; overexpressing NADH dehydrogenase to increase respiration efficiency (Calhoun, Oden, Gennis, De Mattos, & Neijssel, 1993); using engineered enzymes to balance NADH/NADPH generation and consumption (Javidpour et al., 2014); *in situ* product recovery/separation to avoid product inhibitions (Baez, Cho, & Liao, 2011); and first of all, maintaining the optimal cultivation conditions (such as dissolved O_2, substrate concentration, etc.). Further optimization can be achieved on transcription/translation level by using controllable gene expression systems, promoter fine-tuning and RBS optimization (Yen et al., 2015). Based on our results, it can be stated that our major objective, creation of a stable biosynthetic pathway for BDO, has been achieved. Our study combined two phases of experiments: the results obtained by *in silico* methods were taken into consideration throughout *in vivo* experiments. The designed biosynthetic pathway has been implemented into a metabolic model and knock-out strains suitable for growth-coupled BDO production were determined. Under different environmental and genetic conditions, we found very promising *in silico* molar yields. Although the *in vivo* production yield proved to be below *in silico* predictions, comparable (Hwang et al., 2014) or below (Liu & Lu, 2015; Yim et al., 2011) data reported in literature, production can be increased by further strain optimization (promoter fine-tuning, RBS optimization), optimization of fermentation conditions (pH), and by long-term (few months) adaptation experiments. Notably, development of a robust analytical method for 1,4-butanediol has yet to be carried out. In conclusion, we can say that in the presented study we successfully carried out development of a genetically modified strain able to produce BDO from sustainable raw materials, notable from glycerol, which can constitute the basis of developing new biosynthetic processes for high-added value compounds.

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Publications and participations at conferences

A. Papers published in ISI journals

1. **Réka SINKLER**, , Márta Both-Fodor, Emőke Antal, Hunor Bartos, Szabolcs LÁNYI, Ildikó MIKLÓSSY, Metabolic Engineering Of *E.Coli*: Influence Of Genetic Modifications On Physiological Traits, *Studia Babes-Bolyai, Seria Chemia*, **ISSN (Print):** 1224-7154 , 2019, Impact Factor: 0,3
2. Ildikó MIKLÓSSY, Zsolt BODOR, **Réka SINKLER**, Kálmán Csongor ORBÁN, Szabolcs LÁNYI, Beáta ALBERT, *In silico* and *in vivo* stability analysis of a heterologous biosynthetic pathway for 1,4-butanediol production in metabolically engineered *E. coli*, *Journal of Biomolecular Structure and Dynamics*, ISSN: 0739-1102 (Print) 1538-0254 (Online), 1-16, 2016, Impact Factor: 3,123

B. Papers published in journals included in International Databases

Réka SINKLER, Melinda VERÉBI-KERTÉSZ, Dóra NÉMETH, Marianna PAPP, Ferenc KILÁR: Transferrin-iron-anion complex transport into HeLA cells, *Scientific Bulletin-University Politehnica of Bucharest*, Seria B, Chemistry and Material Science, ISSN (print): 1454-2331 / (online): 2286-3680, Vol.82, Iss. 2, 2020

C. Participation in conferences (2014-2019)

1. SALAMON Pál, BOTH-FODOR Márta, **SINKLER Réka**, ALBERT Beáta, SIPOS Emese, MIKLÓSSY Ildikó, Application of RT-qPCR in studies concerning effects of genetic modifications and culture conditions, *23rd International Conference on Chemistry*, Deva, October 25-28, 2017

2. **Réka SINKLER**, Márta Both Fodor, Pál Salamon, Emőke Antal, Zsolt Bodor, Ildikó Miklóssy, Szabolcs Lányi Heterologous expression, purification, and primary characterization of an alcohol/aldehyde dehydrogenase suitable for 1,4 butanediol production, *XXth Romanian International Conference on Chemistry and Chemical Engineering-RICCCE*, Sibiu, September 6-9, 2017, Romania.

3. BODOR Zsolt, MIKLÓSSY Ildikó, **SINKLER Réka**, ORBÁN Kálmán Csongor, LÁNYI Szabolcs, ÁBRAHÁM Beáta, An in silico Re-design of the Metabolism in Escherichia coli for Increased 1,4-butanediol Production from Renewable Feedstocks, 21 st International Conference on Chemistry, Șumuleu Ciuc, September 23-27, 2015, Romania

4. MIKLÓSSY Ildikó, BODOR Zsolt, **SINKLER Réka**, ORBÁN Kálmán Csongor, ÁBRAHÁM Beáta, LÁNYI Szabolcs, Production Possibilities of Non-natural Metabolites by Genetic Engineering, 21st International Conference on Chemistry, Șumuleu Ciuc, September 23-27, 2015, Romania.

5. Zsolt BODOR, Beáta ÁBRAHÁM, **Réka SINKLER**, Szabolcs LÁNYI, Csongor ORBÁN, Ildikó MIKLÓSSY, Fermentation strategies for 1,4-butanediol production from bio-industry byproducts, *3rd European Congress of Applied Biotechnology*, Nice, September 27-October 1, 2015, France.

6. Zsolt BODOR, Ildikó MIKLÓSSY, **Réka SINKLER**, Kálmán Csongor ORBÁN, Szabolcs LÁNYI, Beáta ÁBRAHÁM, *Escherichia coli* metabolizmusanak *in silico* áttervezése megnövekedett 1,4-butandiol előallításához megújuló alapanyagokból (An *in silico* Re-design of the Metabolism in *Escherichia coli* for Increased 1,4-butanediol Production from Renewable Feedstocks), *XXI Nemzetközi Vegyészkonferencia*, Csíksomlyó, Szeptember 23-27, 2015, Románia.

7. Ildikó MIKLÓSSY, Zsolt BODOR, **Réka SINKLER**, Kálmán Csongor ORBÁN, Beáta ÁBRAHÁM, Szabolcs LÁNYI, Nem természetes metabolitok előállítási lehetőségei génsebészeti eljárásokkal (Production Possibilities of Non-natural Metabolites by Genetic

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8. **Réka SINKLER**, Ildikó MIKLÓSSY, Zsolt BODOR, Kálmán Csongor ORBÁN, SZABOLCS Lányi, Beáta ÁBRAHÁM, Critical Analysis of Enzyme Selection, Expression and Production Potential of New Biosynthetic Pathway Enzymes in Different Knock-out Mutants of *Escherichia coli*, *XIXth Romanian International Conference on Chemistry and Chemical Engineering-RICCCE*, Sibiu, September 2-5, 2015, Romania.
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