Growth and metabolite production of *Basfia succiniciproducens* using different substrates

PhD thesis

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Summary

Our modern life is unimaginable without plastics. However, the problems of widely used plastics arise not only during production, but also during degradation or recycling after use. At the moment, the polymers synthesised by the chemical industry are still largely based on fossil fuels, and we also use a significant amount of non-renewable energy sources through waste management. Taking all this into account, it is clear that there is a need to develop and implement methods, both in the production of plastics and in the life cycle of products, that reduce these negative environmental impacts.

Recent rapid advances in biotechnology and high-throughput technologies have introduced new directions that can address the problems listed above, and relieve the burden on the chemical industry.

Succinic acid is such an alternative platform chemical, produced biosynthetically using various microorganisms. Natural succinic acid producer strains are already used in industry include *Actinobacillus succinogenes, Mannheimia succiniciproducens, Basfia succiniciproducens, Saccharomyces cerevisiae* and metabolic engineered *Escherichia coli*.

In the present thesis, various analyses and studies were carried out on the growth and metabolic productivity of the *Basfia succiniciproducens* strain, using the strain in wet experiments and the corresponding *in silico* metabolic model. The research can be divided into two major phases: in the first step, I focused on the extension of the *in silico* metabolic model and the analyses with it, while in the second step, I investigated the metabolic productivity of the strain under laboratory conditions, up to the bioreactor experiments that laid the foundation for the succinic acid production technology. In the wet experiments, a broad substrate spectrum was investigated in microplate experiments. After that, the strain was analysed in larger volumes (flasks). Finally, I collected data on the metabolic profile of the strain in bioreactor, under controlled conditions. As a frame of the research the resulting data from lab experiments were used as feedback, to perform another computational prediction (**Figure 1**). *In silico* analyses shows that by extending the model, quantitative predictions related to succinic acid yield can be computed for the two newly integrated substrates: xylose and glycerol. Laboratory experiments have shown that the bacterial strain has a broad-spectrum substrate utilisation ability, and that the applied gas mixture has a beneficial effect on the biomass and succinic acid production of the strain.

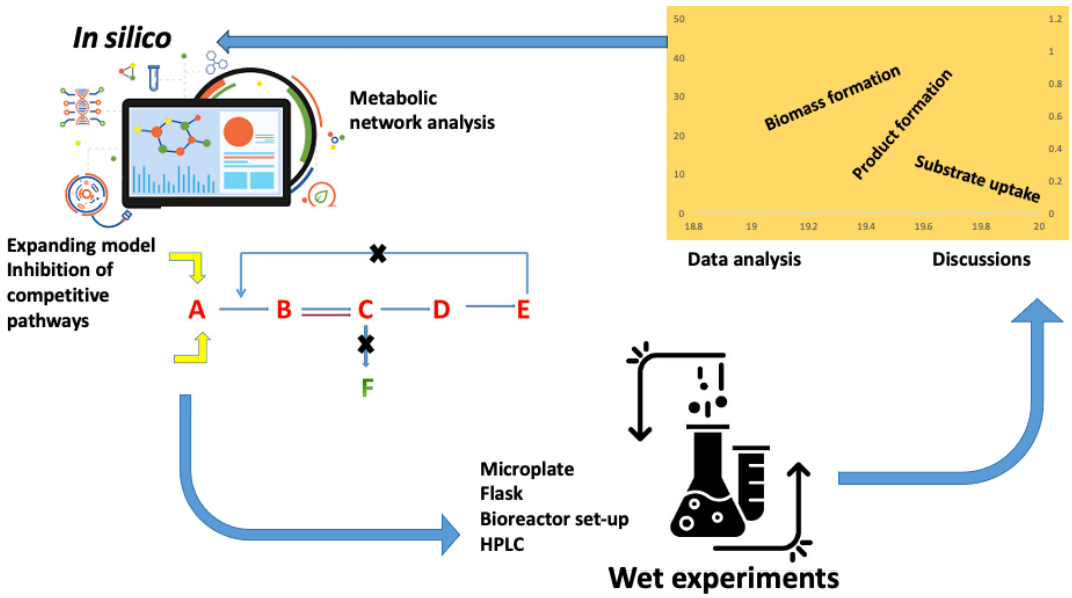

Figure 1. Schematic representation of the PhD research process

1. Introduction: the significance of the study and the hypotheses

Succinic acid, produced by *Basfia succiniciproducens*, can be an alternative solution to the previously outlined problems. Succinic acid is produced by many large companies in the world (BASF, BioAmber, Royal DSM, Reverdia, Succinity) using various microorganisms and carbon sources (Saxena et al. 2017). The global market value of succinic acid in 2021 was more than $158 million (Hariz et al. 2023).

The direction of this PhD thesis is given by the questions listed below:

1. The expansion of the *in silico* model, specialized for the *Basfia succiniciproducens* strain may be suitable for the prediction of succinic acid production based on xylose and glycerol substrates

2. The mentioned bacterial strain has a wider substrate utilisation spectrum than previously described in literature, and shows population dynamics even with the use of a minimal medium

3. The used gas mixture throughout the fermentation process affects the biomass and succinic acid formation

4. Simple *in silico* metabolic models, can be used to determine baseline predictions regarding productivity

2. Objectives

Using renewable energy sources, succinic acid biosynthesized by fermentation processes serves as a platform chemical for the production of various biopolymers. With the development of high-throughput technologies, we have the opportunity to perform computer analyzes and predictions. Knowing the main line of the metabolic network of a given microorganism, a selected parameter can be widely characterized. The main goal of this thesis is to determine the effect of different carbon sources and growth conditions on the biomass and succinic acid production ability of the *Basfia succiniciproducens*. In order to achieve the aforementioned goal, and to answer the hypotheses of the research, we can draw up the following steps:

- to answer the first question, I expanded the metabolic model of the *Basfia succiniciproducens,* which was already created and was available in the literature, to the two interested substrates (glycerol and xylose). *In silico* analyzes with the modified model (flux balance analysis, theoretical maximum and gene deletion) were calculated for data from the literature and from my own measurements
- to prove the second assumption, firstly I examined the effect of the substrates on the growth of the strain in small volume (microplate experiments), and then in a larger volume (flask experiments), in different growth medium
- to answer the third question, I analyzed the growth of the bacterial strain in bioreactor, where I used gas mixes that provide microaerobic and anaerobic conditions
- to answer the fourth suggestion, I used the data from the laboratory experiments to perform another *in silico* analysis

3. Experimental

3.1. *In silico* analysis of the metabolic productivity of the bacterial strain

During the *in silico* analyses, I used the latest available metabolic model of the bacterial strain (Kim et al. 2007). This basic model contains approximately 60 reactions, with

the associated metabolites. In order to increase the biosynthesis of the succinic acid, as well as the ability to utilize a wider range of substrates, I firstly expanded the basic model with new components (substrates: glycerol and xylose), and then with new reactions (enzymes and the metabolic pathways catalyzed by them). The applicable reactions and enzymes for the use of xylose and glycerol were selected, based on literature sources and specific databases (KEGG, ECOCYC, BRENDA, PDB, NCBI) (Sinkler et al. 2019):

- xylose isomerase (EC 5.3.1.5)
- xylulose kinase (EC 2.7.1.17)
- glycerol kinase (EC 2.7.1.30)

The computer analyses were performed with MATLAB (Mathworks Inc., Natick, Massachusetts, United States) and the integrated COBRA Toolbox and Gurobi Optimizer (Gurobi Inc., Ann Arbor, Michigan, United States) software packages. Based on data from literature, the glucose uptake rate was set to 7.7 mM $gCDM^{-1}h^{-1}$. The uptake rates of glycerol and xylose during the simulations were 15.4 and 9.24 mM $gCDM^{-1}h^{-1}$, respectively.

Foremost, the uptake rate of the substrates and oxygen was determined. The substrate uptake rate was set to the previously mentioned values, while the oxygen uptake rate (oxygen = 0 mM $gCDM^{-1}h^{-1}$) was set to zero, thereby anaerobic conditions were created. In the second case, the theoretical maximum of succinic acid formation was examined. This consisted in the limitation of the maximum biomass formation rate (0.1 h^{-1}). For the third time, during the computer analyses, I eliminated the metabolic pathways of pyruvate formate lyase (E.C. 2.3.1.54) and lactate dehydrogenase (E.C. 1.1.1.27), Δpfl and Δldh respectively. Finally, analyses were performed with the metabolic model, using the data from the lab experiments (glucose, glycerol, xylose: 7.89-22.7-12.2 mM $gCDM^{-1}h^{-1}$).

3.2. Examination of the used bacterial strain in wet experiments

The strain was purchased from the German Leibniz Institute (DSMZ), and was analysed in three different set-up. First, the strain wide range substrate ability was examined on microplate experiments, later in larger flask experiments the cell growth was analysed in case of the three carbon sources (glucose, glycerol and xylose), in complex (TSB, BHI) and minimal (M9) mediums. For the third time, under controlled conditions, in bioreactor set-up, the strain's growth and metabolite production was examined.

3.2.1. Wet experiments on microplate

The microplate experiments were performed using Fluostar Optima microplate reader (FluoStar Optima, BMG Labtech, Ortenberg, Germany), for the qualitative and quantitative examination of possible fermentation conditions and nutrients. During the experiment, ten different, potentially applicable substrates were examined, which are the following: arabinose, fructose, glycerol, glucose, inulin, lactose, maltodextrin, maltose, mannose and xylose. To determine the optimal growth concentration, several carbon sources were examined at different concentrations (5-10-15-20-25-30-50-70 g/L). During the experiments, three different mediums (TSB, BHI, M9) were examined. The experimental sequences were performed in three repetitions.

3.2.2. Lab experiments in flask

To collect more information regarding the bacterial strain, a scale-up process was applied. Based on the results obtained from microplate experiments, the initial substrate concentration was set up to 20 g/L. 200 mL of medium was poured into Simax flasks with a total volume of 300 mL (Kavalier, Sázava, Czech Republic) and incubated at 37 °C at 180 rpm in a shaking incubator (Sartorius CERTOMAT®BS-T, Göttingen, Germany).

3.2.3. Bioreactor set-up

After the experiments carried out in flasks, the fermentation processes in a larger volume and more controllable conditions were examined. The circumstances of the bioreactor fermentations were chosen from the flasks experiment. In this case the experiments were carried out in the bioreactor (Sartorius Biostat®A Plus, Göttingen, Germany) using the following carbon sources: glucose, glycerol and xylose. Using 2⁄3 of the total volume (1500 mL) of the bioreactor (1000 mL medium), the initial optical density at 600 nm was set to 0.3 with a Camspec M330 (Spectronic Camspec Ltd., Garforth, Leeds, United Kingdom) spectrophotometer. The initial substrate concentration was 20 g/L, and the pH was set to 7, and it was controlled by software (BioPAT®MFCS, Sartorius, Göttingen, Germany), using 1 M NaOH and 1 M HCl. During the fermentations in bioreactor, two different conditions were tested, one bioreactor operated in microaerobic manner (60/60 mL/min carbon dioxide/air (CO_2/LEV) mixture), while the another was operated under anaerobic conditions (60 mL/min

CO_2). To ensure anaerobic conditions CO_2 was introduced into the headspace of the bioreactor, with a flow of 60 mL/min. In order to prevent the acidification of the medium and the subsequent large amount of NaOH entering the medium, the gas phase was introduced into the headspace of the bioreactors. During the fermentation, samples (5 mL) from the bioreactor were taken every two hours for 12 hours, while the change in optical density was recorded, and then the growth rates from the collected data was calculated.

3.2.4. Sample analysis with high-performance liquid chromatography

Samples collected during fermentation were analyzed by using automated, 1260 Infinity (Agilent Technologies Inc., Santa Clara, California, United States) high-performance liquid chromatography. For analysis of the components found in the samples, Coregel 87H3 column was used, with 50 ℃ operating temperature. RID (Refractive Index Detector) and DAD (Diode Array Detector) detectors were used to detect the carbohydrates and organic acids found in the sample, respectively. Sulfuric acid in 0.004 M concentration was used as mobile-phase with a 0.6 mL/min flow rate. For the analysis and for creating standard curves, solutions with a concentration of 20 g/L in the case of carbohydrates, and 50 mM in the case of organic acids were prepared. After the injection, chromatographic peaks were obtained, and the chromatograms generated in this way were evaluated based on the retention time compared to the standard curves.

3.2.5. Calculations

The specific growth rate was determined using the following linear differential equation **(1)** in the microplate, in the flask as well as in the bioreactor set-up:

$$\mu\, x = dx / dt \quad \textbf{(1)}$$

where, “μ x” denotes the specific growth rate of the bacterial strain, “dx” the concentration of bacteria, and“dt” the elapsed time .

Also, in the case of all three conditions (on microplate, in flask and in bioreactor), the correlation between the optical density and the concentration of cells was used in the following results and discussions. This correlation is specific for the *Basfia succiniciproducens* bacterial strain, as established by Becker and his research group:

gCDM [g/L] = 0.331 X OD600 (Becker et al. 2013)

4. Results

4.1. Expansion of the metabolic model and the prediction of metabolite production

In addition to the biomass growth rate, other metabolites were also formed during the *in silico* predictions: succinic acid, acetic acid and formic acid. The formation rate was expressed in the unit of measure: "mM $gCDM^{-1}h^{-1}$". With a 0.3 h^{-1} biomass growth rate, the formic acid formation rate on a glucose substrate was the highest, with a value of 6.88 mM $gCDM^{-1}h^{-1}$. The uptake rates of xylose and glycerol were set up to 9.24 mM $gCDM^{-1}h^{-1}$ and 15.4 mM $gCDM^{-1}h^{-1}$, respectively. The biomass growth rate was 0.26 h^{-1} and 0.448 h^{-1}, respectively. Examining all three substrates (glucose, xylose, glycerol), the formation rate of formic acid was the highest, while in the case of xylose, its value was 6.97 mM $gCDM^{-1}h^{-1}$, while in the case of glycerol, it was 6.49 mM $gCDM^{-1}h^{-1}$. In the case of all three substrates, among the rates of formation of other organic acids, succinic acid was the lowest (respectively 5.57 - 5.79 - 4.52 mM $gCDM^{-1}h^{-1}$).

Applying constraints (limits) on biomass formation rate (μ=0.1 h^{-1}), succinic acid formation rate was similar in the case of glucose and xylose, 12.06 mM $gCDM^{-1}h^{-1}$. While in the case of glycerol it was slightly lower, 11.72 mM $gCDM^{-1}h^{-1}$.

When the pyruvate-formate-lyase gene was eliminated (*Δpfl*), a decrease can be observed in the biomass growth rate, in case of all three applied substrates. The lowest decrease in biomass growth rate can be observed in the case of glycerol, with a value of 10.5%, followed by xylose with 15.4% and glucose with 16.7%. Thus, in the case of *Δpfl*, when glucose and xylose substrates were used, the rate of succinic acid formation increases by 67.59% and 68.1%, respectively, compared to the basic state (wild type - without mutation). In the case of glycerin, there is a smaller but significant increase, the succinic acid formation rate increased by 64%.

4.2. Analysis of the availability of different substrates, examination of the effect of mediums and substrates on biomass and metabolite production under different conditions

As a result of my preliminary experiments in the laboratory (microplate analyses), all of the substrates used (arabinose, fructose, glycerol, glucose, inulin, lactose, maltose, maltodextrin, mannose, xylose) are suitable for growing the *Basfia succiniciproducens* strain, so 5 g/L than 20 g/L for the applied substrate concentration.

Experiments in the flask showed that the complex TSB medium supplemented with glucose and xylose substrates, resulted in a similar bacterial growth rate. When I used a medium containing glycerol as substrate, I observed a growth phase with a lower slope. Using the complex BHI medium, in the case of all three substrates, I observed a very low slope growth phase (exponential phase). In the minimal M9 medium, using all three substrates, the adaptation phase lasts approximately 0-4 hours, followed by an eight-hour exponential phase characterized by a slight slope.

Regarding the cell growth rate, during the experiments carried out in the bioreactor, in the case of the minimal medium (M9) containing glucose as a carbon source, the anaerobic conditions provided more favorable results. When the medium contained glycerol and xylose the carbon dioxide/air (CO_2/LEV) gas mixture proved to be the most effective condition, the biomass growth rate of the bacterial strain was higher. Regarding the yield of succinic acid, the application of the gas mixture carbon dioxide/air (CO_2/LEV) was more useful in the case of substrates: glucose and glycerol. The medium containing xylose, in the case of anaerobic conditions (CO_2), a higher yield of succinic acid, in the value of 0.277 mol/mol was calculated.

5. Discussion

Using the metabolic model, compiled by several research groups, found in the literature, as a first and important step I extended the reconstruction - metabolic model - to the carbon sources of interest in this research (glycerol, xylose). Based on my best literature knowledge, I was the first one who integrated the metabolic pathways of the utilization of the above-mentioned two carbon sources (glycerol and xylose) into the *in silico* model. Until

now, we have been able to obtain results for the analysis performed in the case of glucose as a carbon source, thanks to Becker (Becker et al. 2013). The expanded model is suitable for predicting the growth rate of biomass as well as the formation rates of various organic acids, which can show a useful connection with practical experiments carried out in the laboratory.

Based on the simulations carried out under computer conditions with the *in silico* metabolic model and their results, I can affirm that it is reasonable to carry out succinic acid production on glycerol and xylose substrates, in a bacterial strain optimized with the elimination of the *pflB* gene, in lab experiments, thus contributing to increasing the yield of the target product, and at the same time to the further use of sustainable substrates.

Based on the results of the microplate and flask experiments carried out in the laboratory, I can declare that nine of the ten investigated substrates proved to be potentially usable carbon sources for the *Basfia succiniciproducens*. According to the literature, Kuhnert and his research group worked on a similar topic, where they investigated the carbon source preference of the bacterial strain (Kuhnert et al. 2010), and as a context of this work, I supplemented the literature results by proving the usability of the following substrates: lactose, maltodextrin and fructose. In addition, many studies are known where, using various plant-based hydrolysates, both biomass and succinic acid formation were observed (Cimini et al. 2016; Maria et al. 2016; Anna et al. 2018). In view of these results, it seems necessary to use renewable raw materials in the form of complex, industrial mediums containing lactose, fructose or maltodextrin in *Basfia*-based biotechnological processes in further research.

During the fermentation in the bioreactor, two different conditions were investigated. In the first case, in order to achieve microaerobic conditions, a mixture of carbon dioxide/air (CO_2/LEV) was introduced into the headspace of the bioreactor, with a flow rate of 60/60 mL/min, while in the second case, only carbon dioxide (CO_2) with a flow rate of 60 mL/min. Using glucose as a carbon source, different results for the two conditions were obtained: anaerobic conditions contributed more to the biomass growth rate of the bacteria. The bacterial strain showed a 53% higher biomass growth rate in the case of the bioreactor sparged exclusively with carbon dioxide (CO_2). When using the other two substrates (glycerol and xylose), the strain showed a higher growth rate under microaerobic conditions. Regarding the yield of succinic acid, the microaerobic conditions proved to be more effective when using glucose and glycerol as carbon sources. Using the initial concentration of glucose carbon source of 20 g/L, the succinic acid yield was 0.43 mol/mol, while in the case of glycerol the yield was 0.184 mol/mol. Using a xylose as carbon source, the yield of succinic acid showed a value of 0.277 mol/mol. The yield of the product, calculated to the substrate, is

similar to the data found in the literature, where succinic acid was biosynthesized with a yield of 0.31-0.93 mol/mol using glucose, glycerol and xylose as carbon sources (Stylianou et al. 2020; Scholten, Renz, and Thomas 2009 ; Mahsa et al. 2019).

In my opinion, the data from the fermentation experiments carried out in the bioreactor set-up, especially the effect of the composition of the gas mixture used on the biomass and target product yield, provide valuable information for the development of biotechnological processes.

6. Thesis points

1. *In silico* model expansion and predictions - the most important cornerstone of the most accurate description of the relationship between the computer model and reality is the creation of the most detailed model, which describes the biochemical networks. The more detailed metabolic model describes a microorganism, the more accurate the complexity of the metabolic pathways it contains, so that computer predictions can be made. To the best of my knowledge, I was the first to integrate the pathways ensuring the utilization of glycerol and xylose into the *in silico,* computer model. The first formulated hypothesis is answered by the following: with the two new substrates integrated into the computer model (glycerol and xylose) and the mathematically described equations of their biochemical use, I defined new points of connection between the computer modeling and reality.

2. The broad-spectrum substrate metabolizing ability of the *Basfia succiniciproducens* bacterial strain - my preliminary experiments revealed that the strain was able to use all, but one of the available carbon sources (inulin). As an answer to the second assumption, I can affirm that based on the experiments carried out on the microplate, the following substrates are suitable for growing the bacterial strain: arabinose, fructose, glycerol, glucose, lactose, maltodextrin, maltose, mannose and xylose. In the case of association of complex (TSB, BHI) and minimal (M9) mediums with carbon sources, I discovered a certain cell growth. When the minimal medium is used, it becomes evident that the strain can’t metabolize inulin effectively. However, the utilization of the other nine carbon sources proves that all of the monosaccharides from different sources are suitable for culturing the strain.

3. The relationship between the bacterial strain and the used gas mixture - as a result of my experiments carried out in a bioreactor, I can conclude that the assumption in order to achieve a higher succinic acid yield, it is necessary to keep the strain in microaerobic conditions during the fermentation period, has gained certainty. When using microaerobic conditions

and, glycerol and xylose as a carbon source, the growth rate of the bacterial strain shows a much higher value (in the case of glycerol and xylose, more than three times and nearly 40% difference compared to anaerobic conditions, respectively). Just as the gas mixture affects the formation of biomass, this can also be observed in the case of the other organic acids produced. In the case of microaerobic conditions and using glycerol as substrate, both the biomass concentration and the yield of succinic acid were much higher (more than three times and five times). When xylose was used, microaerobic conditions proved to be more beneficial in terms of biomass formation as well. Regarding the yield of the target product, anaerobic conditions were more effective.

4. *In silico* and practice - my last hypothesis dissects the topic that even simple models, which do not contain wide details, can be suitable for computing basic predictions. During my research, I carried out *in silico* and laboratory experiments in order to gain the widest possible insight into the metabolism of the *Basfia succiniciproducens* bacterial strain. In the first case, I made predictions with the *in silico* model based on literature data. During my laboratory experiments, I created input parameters for the *in silico* model from the practical data and performed new analyzes with them. Finally, it can be concluded that the analyses performed on new input substrate uptake rates derived from laboratory experimental data show predictions that are close to reality.

7. List of publications

Publications contributing to the preparation of this PhD thesis:

1. Márta Balázs, Hunor Bartos, Szabolcs Lányi, Zsolt Bodor, Ildikó Miklóssy (2023), "Substrate type and CO_2 addition significantly influence succinic acid production of *Basfia succiniciproducens*". Biotechnology Letters, 45(9). **IF:2.7**

2. Hunor Bartos, Márta Balázs, Ildikó Hajnalka Kuzman, Szabolcs Lányi, Miklóssy Ildikó (2021), "Production of High Added-Value Chemicals in *Basfia succiniciproducens*: Role of Medium Composition". Sustainability, 13(6), 3513. **IF: 3.88**

3. Réka Sinkler, Márta Both-Fodor, Emőke Antal, Hunor Bartos, Szabolcs Lányi, Ildikó Miklóssy (2019), "Metabolic engineering of *E.coli*: influence of gene deletions and heterologous genes on physiological traits". Studia UBB Chemia, LXIV(2), 159-174. **IF: 0.49**

TOTAL IF: 7.07

Conference presentations contributing to the preparation of this PhD thesis:

1. Bartos Hunor, Miklóssy Ildikó, Balázs Márta, Albert Csilla, Bodor Zsolt (2019), "Substrate utilization studies of the succinate producer *Basfia succiniciproducens*", 19th International Symposium and Summer School on Bioanalysis, Suior, Romania, Abstract book p.82

2. Bartos Hunor, Miklóssy Ildikó, Balázs Márta, Bodor Zsolt (2019), "Analysis of metabolic potential of *Basfia succiniciproducens* for bio-based succinic acid production from renewable resources", 10th International Conference on Environmental Engineering and Management, Jászvásár, Romania, Abstract book p.41

Conference poster presentations contributing to the preparation of this PhD thesis:

1. Bartos Hunor, Miklóssy Ildikó, Bodor Zsolt, Both-Fodor Márta, Lányi Szabolcs (2018), "*In silico* and *in vivo* investigation of high added-value components production in *Basfia succiniciproducens*", 18th International Symposium and Summer School on Bioanalysis, Komárom, Szlovákia, Abstract book p.50

2. Balázs Márta, Bartos Hunor, Antal Emőke, Miklóssy Ildikó, Bodor Zsolt (2018), "A *Basfia succiniciproducens* bioszintetikus potenciáljának tanulmányozása", XXIV Nemzetközi Vegyészkonferencia, Erdélyi Magyar Műszaki Tudományos Társaság (EMT), Szováta, Románia, Konferenciakötet

3. Bodor Zsolt, Bartos Hunor, Bodor Katalin, Both-Fodor Márta, Orbán Csongor-Kálmán, Lányi Szabolcs, Miklóssy Ildikó (2018), "Quantitative prediction of *Basfia succiniciproducens* metabolic potential, for succinic acid and 1,4-butanediol production, with constraint-based models", International Conference on Mathematical Methods and Models in Biosciences and a School for Young Scientists, Sofia, Bulgária, Abstract book

4. Bartos Hunor, Miklóssy Ildikó, Bodor Zsolt, Both-Fodor Márta (2017), "Anyagcseremérnökségi módszerek alkalmazása 1,4-butándiol bioszintézise céljából *Basfia succiniciproducens* törzsben", XXIII. Nemzetközi Vegyészkonferencia, Erdélyi Magyar Műszaki Tudományos Társaság (EMT), Konferenciakötet p.85

5. Bodor Zsolt, Kuzman-Ildikó Hajnalka, Both-Fodor Márta, Bartos Hunor, Lányi Szabolcs, Miklóssy Ildikó (2017), "In silico modeling and evaluation of *Basfia succiniciproducens* for 1,4-butanediol production from renewable resources", MATHMODEL'17, Mathematical modeling technological and socio-economic processes, Borovets, Bulgária, Proceedings, Issue 1/2017, ISSN: 2535-0978, p.133

Scientific activities other than the completion of this PhD thesis:

1. Bartos Hunor, Salamon Rozália-Veronika, Albert Csilla, Laslo Éva, Orbán Csongor (2022), "Apple vinegar production using wild apple vinegar bacterial consortia", 20th International Symposium and Summer School, Pécs, Magyarország, Abstract book p.92

2. Varga Orsolya, Péter Tünde, Bartos Hunor, Mara Gyöngyvér (2017), "Strukturális szénhidrátbontó baktériumtörzsek jellemzése növényi növekedést serkentő és antagonista tulajdonságaik alapján", XXXIII Országos Tudományos Diákköri Konferencia, Agrártudományi szekció, Mosonmagyaróvár, Magyarország, Abstract book p.236