PhD THESES

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Research on biotechnological/bioconversion methods for production of glycerol derivatives

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1. Introduction

Glycerol is a potential renewable resource, which can be produced also by synthetic way, but its largest amount comes from the rising biodiesel production. The actuality of the glycerol utilization is demonstrated by the fact, that to the end of 2006 500.000 tons of glycerol surpluses were predicted on the European market at the beginning of the year. The utilization of such a large amount of glycerol makes it possible to produce chemicals of high added value in large amounts. This fact together with the renewable character of glycerol has really high utilizable potential.

Glycerol platform comprises several intermediates of the chemical industry that can be obtained from glycerol via simple technology steps. Such compounds are the 1,3- and 1,2-propanediol (propylene-glycol), acrolein, glycerol-acetate, glycerol-carbonate, glycidol etc. Among them 1,3-propanediol and 1,3-dihydroxyacetone play the most important role. Former is produced mostly by synthetic methods with a capacity of more than 100.000 t/yr, and used in the plastic industry. 1,3-dihydroxyacetone is produced by either synthetic or fermentative way for the cosmetic industry.

Among other glycerol utilization methods the main topic of my researches was to develop a new enzymatic way for simultaneous conversion of glycerol to 1,3-propanediol and 1,3-dihydroxyacetone (*Figure 1.*). The process for this method was patented [21]. The theory of the process is, that some microorganisms utilize glycerol with disproportional reactions forming 1,3-dihydroxyacetone (DHA) together with 1,3-propanediol (1,3-PD) while $NAD^+/NADH_2$ coenzymes are regenerated. In living cells, DHA is then phosphorylated and enters into the glycolysis, where several fermentation products are formed (acetic acid, etanol, butyric acid etc.). During an *in vitro* enzymatic bioconversion process the formation of these byproducts can be avoided.

The enzymatic glycerol bioconversion is carried out by three key enzymes: glycerol-dehydrogenase (GDH, EC 1.1.1.6), which oxidizes glycerol with the help of NAD^+ coenzyme; glycerol-dehydratase (GDHt, EC 4.2.1.30.), which catalyzes the exit of one water molecule from glycerol with (or without) the help of coenzyme B_{12} forming 3-hydroxypropionaldehyde (3-HPA); and finally 1,3-propanediol-oxydoreductase (PDOR, EC 1.1.1.202), which reduces the 3-HPA to 1,3-PD while the GDH-reduced $NADH_2$ is reoxidized (*Figure 1.*).

Experiments were carried out for the production of GDH and DHA with

fermentations of *Gluconobacter suboxydans*, which is well known from its wide range of oxidative ability.

The intermedier 3-HPA, is an effective antibiotic. Since its monomer, dimer and hydrated derivatives form a dynamic equilibrium in diluted solution and they can polymerize with each other, HPA is not stable enough for commercial trade, so it is not available in the market Furthermore, it is a toxic intermedier, so its examination was very important during the study of the glycerol metabolism. For this reason, I studied the fermentation and HPA production ability of *Lactobacillus reuteri* cells, which can tolerate HPA even at high concentrations.

I also studied the production of the PDOR key enzyme for the enzymatic simultaneous 1,3-PD/DHA production by a recombinant *Pichia pastoris.* The PDOR enzyme was cloned into a methanogen *Pichia pastoris* yeast, and I examined the production and mostly the purification possibilities of the recombinant enzyme. This work was the first step of a long work, where our goal is to produce all the three key enzymes with more prouductive recombinant microorganisms.

The center of my researches was to study the natural 1,3-PD producer microorganisms *Citrobacter freundii*, *Klebsiella pneumoniae* and *Clostridium butyricum* from the point of view of high enzyme production and enzyme yield, and of the applicability of their enzymes in the glycerol bioconversion.

In order to apply the 1,3-PD producer enzymes later in industrial scale, I had to solve the enzyme production, the analytical assays of the enzymes, the partial purification, the storage, and the technical application itself. The optimization of the process and the scale-up need further research.

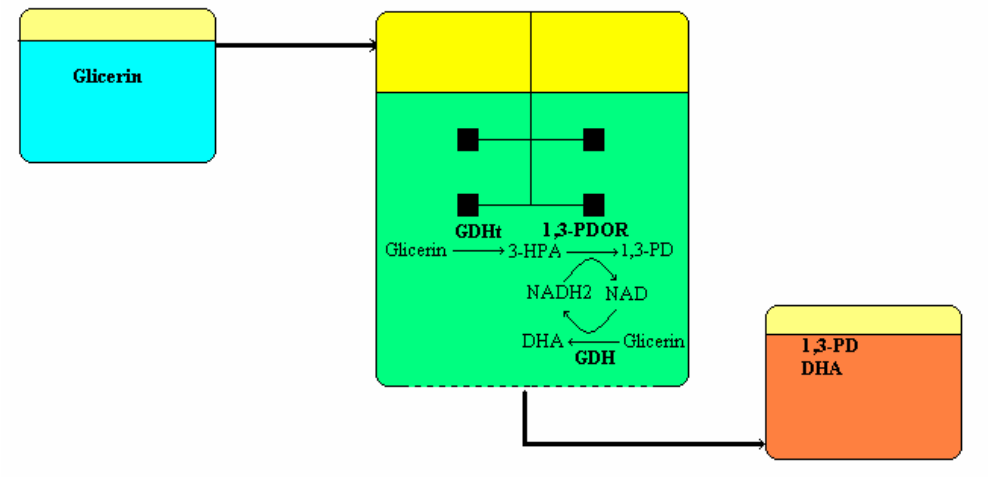


Figure 1.: The scheme of the enzymatic simultaneous 1,3-PD/DHA production

2. Experimental methods

2.1. Fermentation experiments

For the clone selection experiments of the genetically modified *P. pastoris* a spinning test-tube cultivation equipment (Rotomix type 2114 MTA Kutesz) was applied. For the shaking flask experiments of *G. suboxydans, P. pastoris,* and *C. butyricum* cells New Brunswick G10 Gyrotory® and Medicor BRI-1 shaker incubators were used. The small-size fermentations of *G. suboxydans, L. reuteri, P. pastoris, C. freundii, K. pneumoniae* and *C.butyricum* were carried out in a B.Braun Biostat Q DCU fermenters with a working/total volume of 0,8/1L. In the case of *P. pastoris, L. reuteri,* and *C. butyricum* small-size fermentations were also carried out in B.Braun Biostat M fermentor with a working/total volume of 1,5/2L. Larger scale fermentations of *G. suboxydans, P. pastoris* and *C. butyricum* were performed in a B.Braun Biostat U30 20/30L working/total volume bioreactor.

2.2. Bioconversion experiments

The glycerol bioconversion of *G. suboxydans* and *L. reuteri* into DHA and 3-HPA were examined in B.Braun Biostat Q fermentor. For the enzymatic glycerol bioconversion Solvent Resistant Stirred Cell (Millipore) was used, equipped with a 10kDa ultrafilter disk membrane, which retained the enzymes in the reactor during sampling across the membrane. The parallel enzymatic bioconversions were performed in glass flasks of 200 ml total volume with plastic caps, or in plastic flasks with total volume of 50ml. In both cases magnetic stirrer was applied.

2.3. Preparation of cell free crude enzyme solution

Cell culteres were centrifuged in one of the following centrifuges: Seisystem Bio (13000rpm 24x1,5ml), Janetzki T24 (12000rpm, 6x30ml), Janetzki MLW K23D (6000rpm, 4x80ml) or Janetzki KD23 (3000rpm, 4x800ml). For the cell disruption – to release the enyzmes – Techpan UD-11 (150W, Poland) and Labsonic P (300W, Sartorius) ultrasonic

disintegrators were applied. Enzymes were partially purified on an FPLC chromatograph system with different columns (Q-sepharose, Cibacron Blue F-3GA, HiTrap Desalting, HiTrap Chelating HP).

2.4. Analytical methods

The samples of the fermentations and bioconversions were analyzed on Waters Breeze HPLC system with BioRad Aminex HPX87H column on 65°C. The flowrate of the eluent was 0,5ml/min and it contained 0,5mM sulfuric acid in ion-exchanged water. The fermentation samples of *P.pastoris* cell culture containing methanol were analysed on HP Chrompack 438A gas chromatograph equipped with Supelco 0,2% Carbowax 1500 on 80/100 Carbopack C, 6' column. For photometry Pharmacia Ultrospec Plus equipment was used.

2.5. Applied softwares

To determine the background activities during the enzyme activity measurements hyperbolic regression was applied using *SigmaPlot 2001* software. For the statistical evaluation of experimental designs *Statistica for Windows* and *Minitab 14* programs were used. For the mathematical description of the enzymatic glycerol bioconversion and for fitting the differential equation system to the measured data, moreover for simulation studies *Berkeley Madonna 8.01* simulation software was applied.

3. Results

3.1. It was pointed out that GDH enzyme of *G.suboxydans* cells is not suitable for the simultaneous enzymatic production of 1,3-PD/DHA from glycerol.

On the basis of earlier results of our department and other authors [24], *Gluconobacter suboxydans* 621H cells seemed to be an ideal enzyme source, since these bacteria are well known from their wide range oxidative ability. Thus, I drove fed-batch fermentation in 20L scale to get large amount of cell mass containing GDH enzyme . Because the DHA formed from glycerol inhibits the cell growth during the fermentation, sorbitol carbon-source was applied first in a cell growth phase, and then the GDH enzyme was induced by glycerol feed in a second phase. *Figure 2.* shows the characteristic changes in the concentration of dissolved oxygen which assures that both carbon sources (sorbitol and glycerol) was completely utilized, that means GDH enzyme was present in the cells. Since the crude enzyme solution of the harvested cells was not active on glycerol in the presence of NAD^+, a new result of the literature [25] was verified. According to this, the *G. suboxydans* cells have a PQQ-dependent (Pyrroloquinoline quinone) GDH enzyme. Unfortunately in the enzymatic glycerol bioconversion to 1,3-PD and DHA $NADH_2$-dependent PDOR enzyme can only be applied, the PQQ-dependent GDH is not suitable for this process.

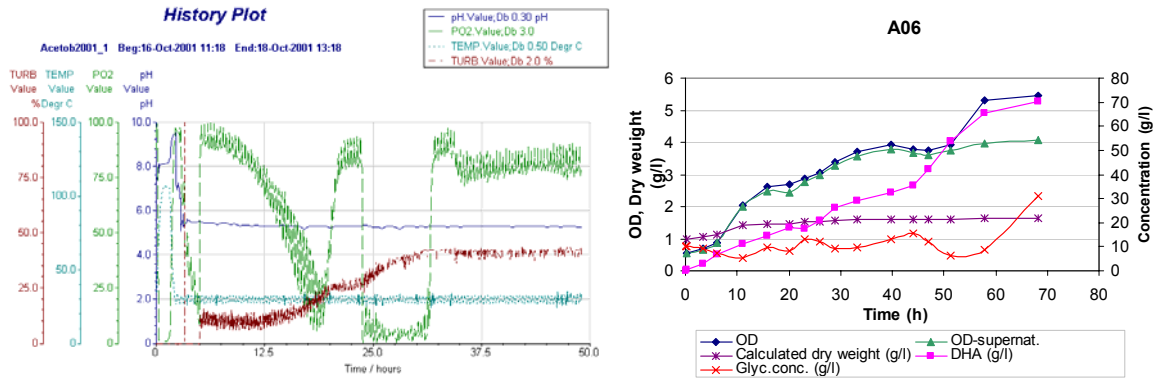


Figure 2.: The sorbitol/glycerol fermentation and DHA formation of *G. suboxydans* cells

At the same time our experiments proved again that DHA can be produced by the fermentation of *G. suboxydans*. Such a DHA fermentation with glycerol feed can be seen on *Figure 2*. On the right hand side of the figure can be seen, that product inhibition arises at around 20g/L DHA concentration and it completes at 70g/L. The DHA yield from

glycerol (70%) and the final DHA concentration (70g/L) seemed to be high enough to use this fermentation process for DHA production after further optimization.

3.2. It can be stated on the basis of the literature and of my own research for glycerol bioconversion to 3-HPA with *L. reuteri* cells, that the most effective method for biomass production of highest bioconversion ability is a batch fermentation method under microaerophilic conditions applying one fresh glucose injection as feed, followed by lyophilisation of the cells.

I intended to produce 3-HPA, which is a promising molecule of the glycerol platform, with fermentation of a *L. reuteri* strain. Since the 3-HPA is a toxic intermedier, the cell growth phase and chemicals production phase have to be separated again. The goal in respect of the cell growth was to produce large amount of good producer cell mass. For this reason, I compared several possible fermentation methods (*Table 1*.).

Table 1. Fermentative production of *L. reuteri* cells

Fermentation	**Condition**	**J_x (g/l*h)**	**J_{xmax} (g/l*h)**	**$Y_{x/s}$ (g/g)**	**$Y_{lacticacid}$**	**Y_{EtOH}**	**gHPA/g d.w.**
LR2006_1	Fed-batch	0,087	0,202	4,2	59,0	36,6	0,048
LR2006_2	Batch(0,7L)	0,092	0,211	6,7	56,6	36,7	0,052
LR2006_3	Batch (1,5L)	0,087	0,087	7,0	57,4	35,6	1,297
LR2006_4	Batch(0,7L)	0,084	0,400	3,6	73,4	23,0	n.a
LR2006_5	Batch(0,7L)	0,093	0,325	4,6	72,7	22,7	n.a
LR2006_6	Batch+1xGlu feed.	0,110	0,321*	5,3	67,4	28,0	4,446
LR2006_7	Batch+1xGlu feed	0,092	0,311	4,4	62,6	33,0	0,014
LR2006_8	Batch(aerobic)	0,028	0,405**	n.a	n.a	n.a	0,399[#]
LR2006_9	Batch(aerobic)	0,023	0,177***	3,8	53,5	42,6[##]	0,323
Literat. [26]	Batch(anaerobic)						12,58g/30g= 0,42
Literat. [27]	Batch(anaerobic)						5,2g/4g=1,3

* biomass production before glycerol injection

**under aerob conditions, S_0=120 g/L, only 40 g/L glucose consumption, insufficient N-source;

***under anaerobic conditions, $S_{0,Glucose}$=20 g/L, $S_{0,Glycerol}$=2 g/L

[#]high activity in bioconversion

[##]acetic acid yield

Among the presented methods in the *Table 1*., the grey marked batch fermentation with one glycerol injection (as feed) was the most effective both from the point of view of 3-HPA producing ability and of biomass productivity (J_x).

For harvesting and treating the produced cells I compared also different methods (*Table 2.*)

Table 2. Comparison of various downstream methods of *L. reuteri* cells

Fermentation	**treatment method**	**specific HPA production (g HPA/g d.w.)**		**Difference**
		Before(free cells)	**After (treated)**	
4,5	Perlit	-	0,236	
4,5	Spray dried	-	0,011	
4,5	gelatin	-	0,0	
6	Phosphate buffer	4,446	0,0	
6	Immobilized on perlit after bioconversion	4,446	0,013	0,3%
7	Lyophilized 1,4ml	0,014 (wrong)	5,963	
7	Lyophilized 10ml	0,014 (wrong)	0,792	
8	Chitosan microcapsule	0,086*	0,557	648%
8	Chitosan macrocapsule	0,399	0,080	20%

It can be stated that – although the measured activity before the treatment was not correct (because the maximum HPA concentration could not be reached) – lyophilization was an excellent treatment. Furthermore, the cell immobilization into chitosan capsule, which is probably cheaper, could also be an effective cell treatment method.

It should be mentioned, that the bioconversions in Eppendorf tubes (1,4ml) gave higher activity as the same preparations in larger (10ml) test tubes (see *Table 2.*, lyophilized rows). The reason was probably the better anoxic conditions in the smaller tubes.

3.3 It can be stated, that the genproduct of the *C. freundii* PDOR gene cloned into *P. pastoris* can not be successfully purified with Ni^{2+} affin chromatography, but can be purified on Q-Sepharose column.

After the screening work of the genetically manipulated *P. pastoris* clones with the best PDOR producer fed-batch fermentation was performed in 20L scale. In the first phase, the

cells were grown on glycerol, which was followed by an adaptation phase and an expression phase on methanol as carbon-source. Cells were harvested by centrifugation and crude enzyme solution was obtained by ultrasonic disintegration followed by centrifugation of cell debris. The genetic modification included a His_6-tag building onto the C'-terminal of the recombinant PDOR, because this way usually allows a selective protein purification on chelat-column binding Ni^{2+} (HiTrap Chelating). In contrast to the expectations my results showed that the recombinant PDOR can not be purified through this way, but at the same time I could partially purify the protein on a Q-Sepharose column. This method was known from the literature for the purification of natural PDOR. The chromatogram of the successful purification can be seen on the *Figure 3*. The recombinant PDOR activity can be found in the 9th peak.

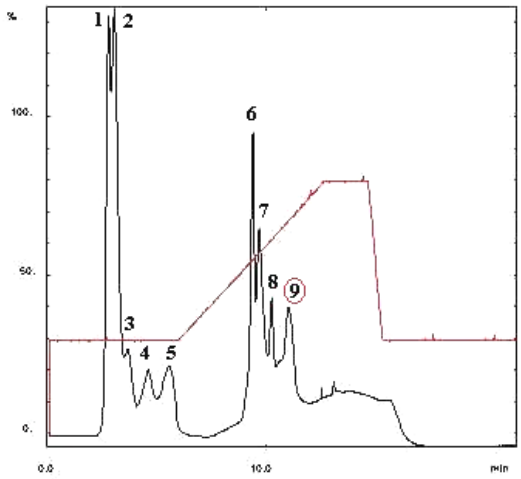


Figure 3. Purification of recombinant PDOR

3.4 It was found, that for the production of *K. pneumoniae* cells in large amount the most effective method is the following: 1. batch fermentation on glucose under aerobic conditions, 2. glucose feed under aerobic conditions, 3. glycerol feed under aerobic conditions, 4. glycerol feed under anaerobic conditions. Taken into account the catbolite repression, the aerated glucose and aerated glycerol phases can be coupled with each other.

The GDHt and PDOR enzymes for the 1,3-PD and DHA production were produced by *C. freundii* and *K. pneumoniae* fermentations. Batch and fed-batch fermentations were carried out under anaerobic conditions on glycerol as carbon-source. *K. pneumoniae* was found more effective with both fermentation techniques, because it reaches higher cell concentration and

enzyme activity. The fermentation process was further developed for *K. pneumoniae* taking into account its facultative anaerobic metabolism and the catabolite repression (*Table 3.*).

Table 3. Comparsion of the fermentation of *C. freundii* and *K. pneumoniae*

Fermentation	**Conditions**	**Cell growth [measured ΔOD_{600}]**	**PDOR activity [U/l broth]**	**1,3-PD [g/l]**
C3	Batch, anaerobic, glycerol	0,1	0,0017	0,114
K1	Batch, anaerobic, glycerol	4	0,098	13,48
K4	4 phase: Glucose (aerobic), Glucose feed (aerobic), Glycerol feed (aerobic), Glycerol feed (anaerobic)	22,88	90	0,1
C6	3 phase: Glucose+Glycerol (aerobic), Glycerol feed (aerobic), Glycerol feed (anaerobic)	7,5	0,005	2
K9	3 phase: Glucose+Glycerol (aerobic), Glycerol feed (aerobic), Glycerol feed (anaerobic)	13,695	73	12
Literature	[28]:0,7L fermentor,batch [29]:2L shaking flask, batch [30]:2L fermentor, continuous	8,26* [30]	37,5 [29]	9,12 [28]

*calculated from cell dry weight

3.5. Although the crude enzyme solution of *K. pneumoniae* contains all necessary key enzymes for the glycerol bioconversion to 1,3-PD/DHA, according to *in silico* results obtained with my complex mathematical model, it is not suitable for the planned process because of the suicide inactivation of GDHt known from the literature [30].

The crude enzyme solution obtained from *K. pneumoniae* culture were used in successful glycerol bioconversions to 1,3-PD/DHA. Since crude enzyme solutions were applied, the formed DHA reacted further to acetic acid. To eliminate the byproduct formation, a mathematical model was established on the basis of enzyme kinetics [7]. The reactions with one substrate were taken into account with Michaelis-Menten kinetics, and the multisubstrate (one substrate and one coenzyme) reactions were described with random bi-bi mechanism. The kinetics of the GDH enzyme was modeled as ordered bi-bi mechanism according to the literature [32]. Furthermore the suicide inactivation of the GDHt enzyme was described with first order decay kinetics and the ATP-dependent regeneration was also modeled according to

the literature. For the activity loss of GDHt the coenzyme B_{12} is responsible, since it forms an inactive complex with the enzyme after each glycerol molecule transformation [31]. My simulations demonstrated, that the acetic acid forming pathway should not be eliminated [11], because then the ATP forming way also stops and as a consequence GDHt can not be regenerated. If it is not possible to regenerate the GDHt key enzyme, then there will be no more 3-HPA for the PDOR, and the 1,3-PD production will be also stopped, which eliminates the regeneration of the coenzyme $NADH_2$ and the DHA formation will be also stopped (*Figure 4.*) [2, 12,].

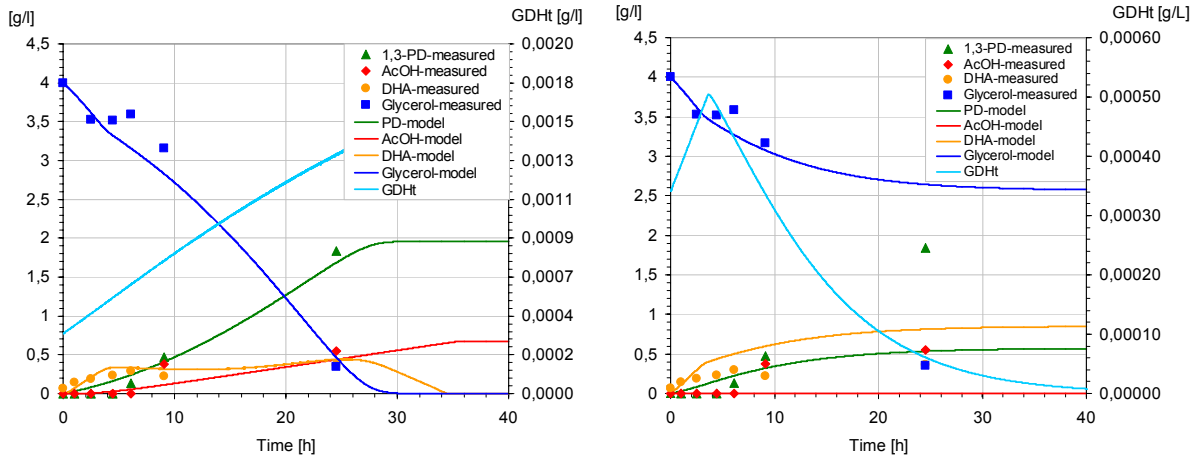


Figure 4. Measured data and simulated results of the bioconversion by crude enzyme solution of *K. pneumoniae* cells with and without DHA phosphorylation

3.6. The crude enzyme solution of *C. butyricum*, in which the GDHt enzyme is B_{12}-independent, is suitable for the simultaneous 1,3-PD/DHA bioconversion of glycerol. This was verified both experimentally and with simulations (*in silico*).

Crude enzyme solution of *C. butyricum* was obtained by ultrasonic disintegration of cells containing all of the three key enzymes (GDH, GDHt, and PDOR). This cell-free extract was used as enzyme solution for several consecutive bioconversions [13], but instead of DHA formation butyric acid was formed. A mathematical model similar to the former one was built to examine the possibility of the byproduct elimination [14]. The one-substrate reaction was described with Michaelis-Menten kinetics, and those using one substrate and one coenzyme (multi substrate) were modeled with random bi-bi mechanism. The simulations certified the reality of the simultaneous 1,3-PD/DHA production with enzymatic glycerol bioconversion

when inhibiting DHA-phosphorylation which eliminates butyric acid formation (*Figure 5.*). This model was used for further simulation experiments.

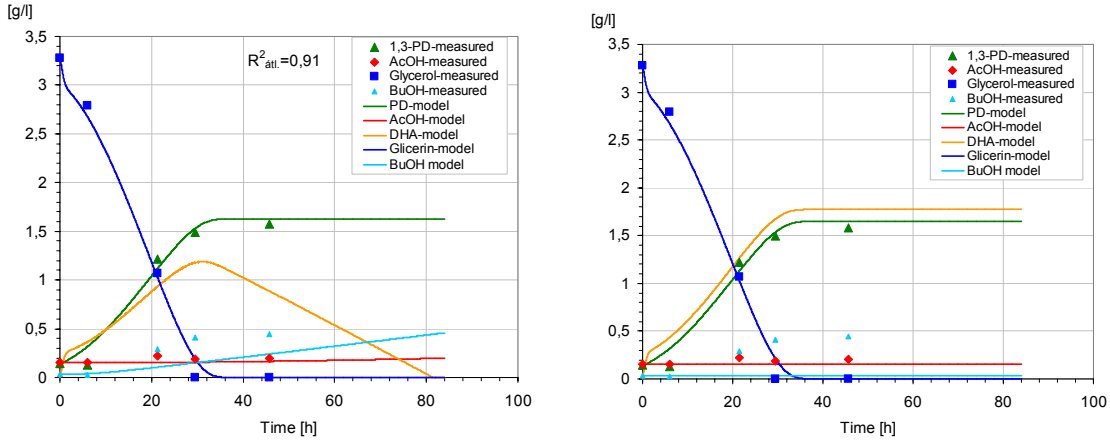


Figure 5. Measured data and simulated results of the bioconversion by crude enzyme solution of *C. butyricum* cells with and without DHA phosphorylation

3.7. On the basis of the verified simulation model it can be stated, that the developed simultaneous 1,3-PD/DHA production with enzymatic glycerol bioconversion can be competitive to the other biological 1,3-PD production process already in operation.

The first „BioPDO" plant started in the year 2006, in which 1,3-PD is produced biologically for the plastic industry (Poly-Trimethylene-Terephtalate, PTT). In this technology recombinant *Escherichia coli* is used for fermentation. Into this host the genes responsible for glucose – glycerol bioconversion were cloned from *Saccharomyces* and the genes for glycerol – 1,3-PD bioconversion (*dha* regulon, containing the genes of PDOR and GDHt) from *C. freundii.* Since the host *E. coli* is not able to synthetise coenzyme-B_{12} *de novo*, it has to be added to the fermentation broth, which elevates the costs. During the development of this new strain the selection was directed to reach high 1,3-PD concentration tolerance as well as the extracellular metabolites were eliminated. Thus during c.a. 2 day fermentation produces ~100g/L 1,3-PD which is equivalent to a volumetric productivity of ~2 g/L*h. Simulations for continuous operation with the mathematical description presented in the previous thesis point were used to examine the effect of the glycerol concentration in feed and the effect of enzyme concentration on the efficiency. This can be seen in the Fig 6. ($P=J_{PD}$/Res.glic, where P is the efficiency, J_{PD} is the volumetric productivity of 1,3-PD, and Res.Glic is the residual glycerol concentration) [15].

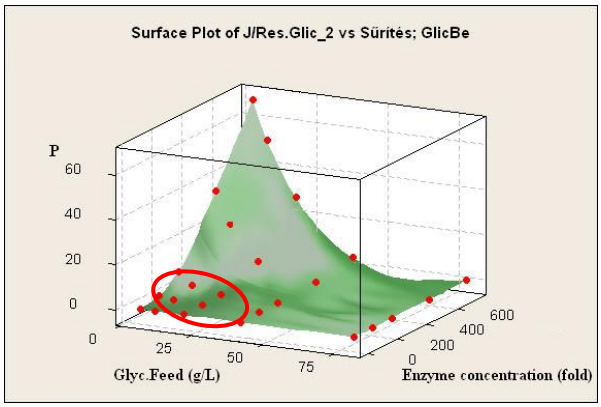


Figure 6. The efficiency of the continuous operation enzyme reactor (at the marked points: JPD>2,5g/L*h and Res.Glic<1g/L)

From these results it is convincing, that the simultaneous 1,3-PD/DHA production can be competitive to the presently used fermentative technology.

3.8. Comparing the crude enzyme solution of *K. pneumoniae* and *C. butyricum* it can be stated, that proportions of the three key enzymes are balanced in the *C. butyricum* owing to the absence of the aerobic/anaerobic change during the enzyme fermentation. In the case of *K. pneumoniae* the amount of GDH is two orders of magnitude higher than the others. The reason is most probably the existence of the glycerol utilizing phase which was performed under aerobic conditions, where GDH is the most needed key enzyme for the cells metabolism.

The proportions of the key enzymes in the crude enzyme solution obtained from optimized fermentation of the two bacteria are compared in *Table 4*.

Table 4. Comparison of the enzyme yields

U/L broth	***K. pneumoniae***	***C. butyricum***
PDOR	14,9	2,1
GDH	1366,6	13,5
GDHt	85,0*	3,3*

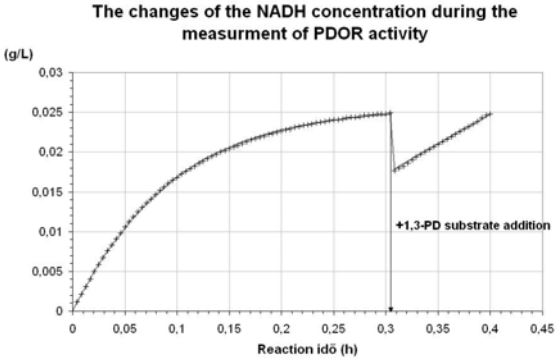
*1,2-PD as substrate was applied during the assay

It can be stated, that enzyme solution of *C. butyricum* contains also mostly GDH, but the amount of this enzyme is only one order of magnitude higher than the other two, while in the case of *K. pneumoniae* GDH concentration is two orders of magnitude larger than the other two enzymes. It should also be mentioned, that these enzyme solutions were used in

bioconversion experiments resulting quite the same accomplishments (*Figure 4.* and *Figure. 5.*) despite the higher activity of all key enzymes in the enzyme solution of *K. pneumoniae.* Since there was no ATP addition, the reason is probably that the ATP-dependent regeneration of GDHt was limited lowering the efficiency of the high activities.

3.9. On the basis of an analytical method development it can be stated, that disturbing minor waste components of crude enzyme solution origin can only be eliminated during the determination of NAD-dependent enzyme activities, if at first the decreasing background activity is measured, and after it disappears, the substrate can be given to the reaction mixture in order to determine the real key enzyme activity.

The assay method for PDOR known from the literature [29] had to be modified, because the presence of the remaining metabolites and coenzymes after removing cell debris caused unwanted enzyme reactions (i.e. reduction of NAD^+) in the examined crude enzyme solution and these disturbed the activity measurements. The activity determination is carried out against the physiological direction (1,3-PD->3-HPA instead of 3-HPA->1,3PD) according to Lin's method. Since in this direction NAD^+ is reduced to $NADH_2$, which can be determined photometrically, the residual glycerol from the fermentation broth disturbs the measurement, because glycerol is converted to DHA by the present GDH and this reaction also forms $NADH_2$. Since during the fermentations to maintain glycerol concentration between 10-16 g/L is advantageous, glycerol always appears in the crude enzyme solution. For this, it is preferred to let the waste (and other) components react (="background reaction measurement") first and later with addition of the 1,3-PD substrate the real determination of the PDOR activity can be started (*Figure 7.*) [3].



$$\text{Enzym activity}\left[\frac{\text{U}}{\text{ml}}=\frac{\mu\text{mol}}{\text{min}\cdot\text{ml}}\right]=\frac{\frac{\Delta A_{lin}-\Delta A_{exp}}{\Delta t\,[\text{sec}]}\cdot 60}{6{,}220\left[\frac{1}{\frac{\mu\text{mol}}{\text{ml}}\cdot\text{cm}}\right]}$$

Figure 7. Improved PDOR activity determination

For the quantitative determination of PDOR activity it is preferred to let the „background activity" disappear and the absorbance changes stop before the substrate addition. In this case, in the equation of *Figure 7*. ΔA_{exp} =0 should be used. If the „background" does not stop, than an exponential function rising to a limit value should be fitted to the points measured without substrate addition. To the absorbance points determined with substrate presence can be fitted a straight line and its slope contains both activities (background and PDOR). With the extrapolation of the exponential curve to the time range of the linear curve the „background activity" can be estimated. With this the slope of the straight line has to be lowered.

4. Possibilities of application

As a result of my researches I worked out a process for the simultaneous 1,3-PD/DHA enzymatic production from glycerol, which was patented in 2005. [21].

I successfully developed an effective way for 3-HPA (antibiotic agent) production.

I selected successfully the most appropriate strain among 4 potential enzyme sources to produce the key enzymes for the enzymatic bioconversion of glycerol to 1,3-PD/DHA.

On the field of enzyme analysis new innovations were introduced, which are essential for the researches above, but which can be applied also over my researches.

5. Publications in the field of the dissertation

Articles in edited journals

1. Németh, Á.; Kupcsulik, B.; Sevella, B., 1,3-Propanediol oxidoreductase production with *Klebsiella pneumoniae* DSM2026. *World Journal of Microbiology and Biotechnology* **2003,** 19, (7), 659-663.

2. Németh, Á.; Sevella, B., Development of a New Bioprocess for Production of 1,3-propanediol I.: Modeling of Glycerol Bioconversion to 1,3-propanediol with Klebsiella pneumoniae Enzymes. *Applied Biochemistry and Biotechnology* **2008**. 144(1). 47-58. DOI: 10.1007/s12010-007-0040-5

3. Németh, Á.; and Sevella, B., Difficulties and solutions for the assays of the key enzymes of a new enzymatic glycerol bioconversion. *Periodica Polytechnica* **2008,** accepted

Articles in non edited journals

4. Németh, Á.; and Sevella, B., Research on the utilization of the biodiesel byproduct glycerol. *Hungarian Journal of Chemistry* **2007,** 113 (2), 58-61

Oral presentations

5. Németh, Á., Kupcsulik, B. and Sevella, B., 1,3-propanediol dehydrogenase production with Klebsiella pneumoniae. *HAS Complex Committee on Food Science, Hungarian Scientific Society for Food Industry and Central Foodscience Research Institute 307. Scientific Kollokvium* **2002,** Budapest, 3.

6. Németh, Á.; és Sevella, B., Research of biotechnological/bioconversional methods for productions of glycerol derivatives *First Conference of PhD students at Faculty of Chemical Engineering* **2003,** Budapest, 78-79.

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