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IMMOBILIZED ENZYMES AVAILABILITY FOR GLYCEROL – 1,3 PROPANEDIOL BIOCONVERSION

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The increasing abundance of glycerol, its renewability, and attractive pricing make it an appealing platform chemical. Glycerol can be utilized in various ways of biological transformation to industrially valuable products. There are some important derivatives among these such as 3-hydroxypropionaldehyde ((3-HPA) commercial name: reuterin), dihydroxy-acetone (DHA), and 1,3-propanediol (PDO). PDO has the largest potential need. Several bacteria are able to ferment glycerol to PDO under anaerobic conditions. A new recombinant technology was worked out with the leadership of DuPont and Genecor in the last decade, in which a recombinant *Escherichia coli* converts glucose directly to PDO. Microbiological production of a molecule has always lower yield than an enzymatic method and usually produces a lot of by-products that can be avoided using enzymatic bioconversion. For years we have been working on a new enzymatic process in order to produce PDO and DHA simultaneously from glycerol. For this enzymatic way three key enzymes are needed: Glycerol-dehydratase (GDHt), 1,3-propanediol-oxydoreductase (PDOR), glycerol-dehydrogenase (GDH). These enzymes must be produced by microbial fermentation. We would like to present the results of developing the fermentation media in economic aspect. Our pleriminary results (not publicated) indicated that the 2xYT medium is perfect for our Clostridium strain. However in economic aspect the concentrations of yeast extract and bactotryptone are too high to scale-up this fermentation procedure. The first purpose of this study was to optimize the fermentation media. Optimized media concentrations are yeast extract, 6 $g \cdot l^{-1}$ and bactotryptone, 2 $g \cdot l^{-1}$ (instead of 10 and 16 $g \cdot l^{-1}$ respectively). The other aim of our work was to develop immobilized enzymes suitable for glycerol to propanediol bioconversion and develop a method to measure the immobilized enzymes activity. We tried a covalent method applying chitosan matrix activated with glutaraldehyde. Chitosan – a natural polyaminosaccharide obtained from chitin – was chosen as support material to bind the three key enzymes from the crude enzyme solution. We could detect that PDOR and GDH enzyme bind to chitosan beads. Immobilized enzymes activity was higher than of the soluble enzymes.

Keywords: Glycerol; 1,3-propanediol; enzyme activities; immobilization

Introduction

As glycerol is a nontoxic, edible, biodegradable compound, it will provide important environmental benefits to platform products. Glycerol can be converted into industrially valuable derivatives through various ways of biological transformation. There are some important derivatives among these such as 3-hydroxy-propionaldehyde (3-HPA), dihydroxyacetone (DHA) and 1,3-propanediol (PDO).

PDO has the largest potential with more than 100 000 t/yr world production capacity. Two examples of large scale applications of 1,3-propanediol are SORONA made by DuPont applying BioPDO produced via biological way and CORTERRA [1], trade mark of Shell utilising terephtalic acid (PTA) and PDO produced by chemical synthesis (*Fig. 1*).

A new recombinant technology [2] was worked out with the leadership of Dupont and Genencor in the last decade, in wich a recombinant *Escherichia coli* converts glucose directly to PDO. Although the fermentative PDO production of DuPont is much more environmental friendly than the chemical way, an enzymatic process would be more advantageous. It would not have an upper limit of product yield (which is also decreased by the biomass formation), and would not produce any unwanted metabolites. That is why industrial enzymatic process is usually called "Clean technology".

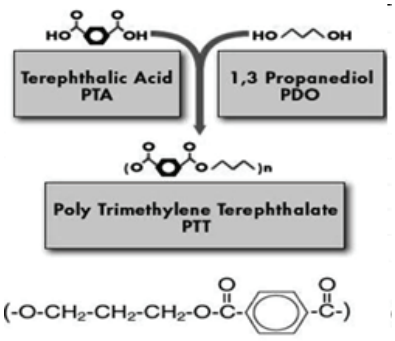

Figure 1: Basic unit of CORTERRA

Our research group has been working for several years on a process [3], where glycerol is converted into PDO and DHA simultaneously by the enzymes of *Clostridium butyricum*.

For this enzymatic way three key enzymes are needed [4]. Glycerol-dehydratase (GDHt; E.C.4.2.1.30) that produces 3-HPA from glycerol with or without B_{12} as coenzyme (our *Clostridium butyricum* origin enzyme is B_{12}-independent); 1,3-propanediol-oxydoreductase (PDOR; E.C.1.1.1.202) that converts HPA into 1,3-PD using $NADH_2$.

In our process the NAD^+ produced by PDOR can be regenerated by a series of oxydoreductases (alcohol-dehydrogenase, formate dehydrogenase etc.), but we usually use glycerol-dehydrogenase (GDH, E.C. 1.1.1.6) which produces DHA (as the third key enzyme).

Anaerobic conditions (Biebl et al. 1999). *Clostridium butyricum* is to our knowledge the best natural 1,3-producer from glycerol [6] and the only microorganism identified so far to use a coenzyme B_{12}-independent glycerol dehydratase. Our pleriminary results (not publicated) indicated that the 2xYT medium described by Reynauld et al [7] is perfect for the Clostridium strain and suitable for enzyme production. However from economic aspect the concentrations of yeast extract and bactotryptone are too high to scale-up this fermentation. One of the purposes of this study was to optimize the fermentation media.

Immobilization has been revealed as a very powerful tool to improve almost all enzyme properties, if properly designed, like stability, activity, specificity and selectivity, reduction of inhibition effects. Immobilized biocatalysts offer some further advantages, like the reusability of the enzymes, the availability of the product in higher purity.

Chitosan is known as an ideal support material for enzyme immobilization because it is non-toxic, and not harmful to the enzymes. In addition to these, chitosan appears economically attractive since chitin is the second most abundant biopolymer in nature beside cellulose.

To carry out enzymatic bioconversions, we have already used successfully a membrane reactor system retaining the enzymes [8-9]. To compare it to other immobilization technics we tried a covalent immobilization method with chitosan matrix. The other aim of our work was to develop immobilized enzymes suitable for glycerol to propanediol bioconversion.

Materials and methods

Organism and medium

Clostridium butyricum NCIMB 8082 (the same as VPI 1718) was used as enzyme source. The strain were stored at 4 °C on Petri dish on modified Reinfored Clostridial Medium (Difco, agar: 12.5 g). Batch cultures of *C. butyricum* were grown anaerobically at 37 °C in 2xYT medium (yeast extract, 10 $g \cdot l^{-1}$; bactotryptone, 16 $g \cdot l^{-1}$; NaCl, 5 $g \cdot l^{-1}$), supplemented with 20 $g \cdot l^{-1}$ glycerol or glucose as carbon source [5]. However, as described under "Results", the concentrations of yeast extract, bactotryptone, glycerol and glucose were varied.

Analytical methods

The cell concentrations were estimated as cell dry weight using a predetermined correlation factor (K: 1.496 $g \cdot l^{-1}$) between optical density at 600 nm (Pharmacia LKB-Ultrospec Plus spectrophotometer, Pharmacia Co., USA) and cell dry weight.

Substrates and products concentrations were determined by Waters Breeze HPLC System described previously [10].

Fermentation experiments

Batch cultivations are described previously [10, 12] (shown in *Table 1*). Samplings (in the cases of test tubes) were realized from different parallel test tubes at the corresponding timepoint, because the need of anaerobic cultivation performed in a closed ANAEROSTAT system (Merck). The growth temperature was 37 °C, the agitation speed was 200 rpm.

Preparation of cell-free extracts

Fermentation broths (from 1-l Bioreactor (B. Braun Biostat® Q DCU) and 2-l Bioreactor (B. Braun Biostat® M)) were centrifuged at 6000 rpm for 40 min. For resuspending the harvested cells, HEPES buffer pH = 7.4 was applied. This centrifugation/resuspendation cycle was repeated twice.

Cells were disrupted by ultrasonic desintegration (Labsonic-P, 300W; Sartorius) [10]. The enzyme activity of supernatant was determined.

Enzyme assays

1,3-propanediol-oxydoreductase (PDOR), glycerol-dehydratase (GDH) and glycerol-dehydratase (GDHt) activity was determined photometrically using the method described previously [13].

Immobilization method

Chitosan was chosen as support material and enzyme solution including the three enzymes was applied as enzyme mixture. Support was prepared by dropping aqueous chitosan solution into 1 N NaOH solution containing 25% ethanol. Cross linking was carried out with 5% glutaraldehyde in K-phosphate buffer pH 7 (shaked in room temperature, 4 hours).

Immobilization of the enzymes was performed by shaking the activated chitosan beads with enzyme solution at 10 °C (pH 7.4; support/enzyme solution = 1:2). However, as described under "Results", the time of immobilization were varied. At first chitosan beads are white, after cross-linking green and in the end of immobilization yellow.

Results

Fermentation process development

To carry out enzymatic bioconversions a large amount of enzyme solution and high enzyme activity is needed. For this, it was neccessary to eliminate the economical bottleneck (costly media) of the scale up process and to select sufficient carbon source and pH regulation.

The concentrations of yeast extract and bactotryptone are too high to scale-up this fermentation procedure. At first fermentation experiments were made in shaked test tubes and shaking flasks to determine the optimal concentrations (data shown at CHEMPOR2008 [10]). Carbon source was glucose or glycerol, pH regulated with K-phosphates or $CaCO_3$. Beside the maximum level of bactotryptone (BT; 16 $g \cdot l^{-1}$) and two types of substrates we analyzed the effect of yeast extract (YE) concentration (*Fig. 2a*). Biomass production decreased significantly below 6 $g \cdot l^{-1}$ YE. While BT concentration was reduced to 4 $g \cdot l^{-1}$ at constant YE (10 $g \cdot l^{-1}$), the biomass was not fallen remarkably (*Fig. 2b*).

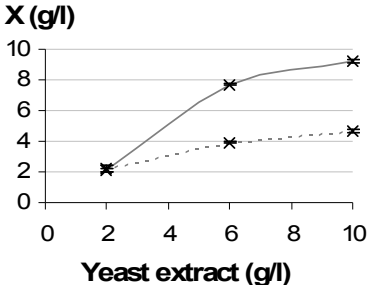

Figure 2a: −x− substrate: 20 g/l Glucose
···x·· substrate: 20 g/l Glycerol

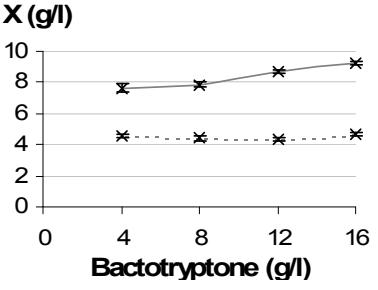

Figure 2b: −x− substrate: 20 g/l Glucose
···x·· substrate: 20 g/l Glycerol

Further fermentation experiments resulted that the BT concentration can be reduced to 2 $g \cdot l^{-1}$ beside pH regulation with $CaCO_3$ [13]. Optimized media concentrations are yeast extract, 6 $g \cdot l^{-1}$ and bactotryptone, 2 $g \cdot l^{-1}$ with glycerol as carbon source and pH regulation with $CaCO_3$.

For enzyme production we carried out several fermentations in lab scale bioreactor. 1,3-propanediol fermentation scale-up procedure results obtained on glycerol batch cultivation with optimized YE and BT are shown in *Table 2*.

*Table 1:*Fermentation experiments scale-up (reference)

Runs	Test type	YE ($g \cdot l^{-1}$)	BT ($g \cdot l^{-1}$)	Glu ($g \cdot l^{-1}$)	Gly ($g \cdot l^{-1}$)
Ferm.1	BQ (1 l)	10	16	20	20
Ferm. 2	BQ (1 l)	10	16	-	20

Runs	X ($g \cdot l^{-1}$)	Y_{PD} ($mol \cdot mol^{-1}$)	Y_{BP} ($g \cdot g^{-1}$)	J ($g \cdot l^{-1} \cdot h^{-1}$)
Ferm.1	6.87	0.57	0.24	0.3
Ferm. 2	7.94	0.61	0.28	0.4

pH regulation: 20% NH_4OH and 25% H_2SO_4
BQ (1 l): 1 l Bioreactor (B. Braun Biostat® Q DCU)
YE: yeast extract; BT: bactotryptone; Glu: glucose;
Gly: glycerol
PD: 1,3-propanediol; BP: by products

*Table 2:*Fermentation experiments scale-up (optimized)

Runs	Test type	YE ($g \cdot l^{-1}$)	BT ($g \cdot l^{-1}$)	Gly ($g \cdot l^{-1}$)
1	SF(400)	6	2	20
2	BQ (1 l)	6	2	20
3	BM (2 l)	6	2	20

Runs	X ($g \cdot l^{-1}$)	Y_{PD} ($mol \cdot mol^{-1}$)	Y_{BP} ($g \cdot g^{-1}$)	J ($g \cdot l^{-1} \cdot h^{-1}$)
1	4.99	0.90	0.42	0.7
2	7.08	0.99	0.34	0.5
3	8.99	0.96	0.28	0.9

pH regulation: $CaCO_3$, 15.489 $g \cdot l^{-1}$
SF: shaking flask
BQ (1 l):Bioreactor (B. Braun Biostat® Q DCU)
BM (2 l): 2 l Bioreactor (B. Braun Biostat® M)
YE: yeast extract; BT: bactotryptone; Gly: glycerol
PD: 1,3-propanediol; BP: by products

All the features of optimized fermentations are better than results of reference fermentations shown in Table 1. Reference enzyme activities from Ferm. 1. and Ferm. 2. are shown in *Table 3*. Enzyme activities and protein concentrations from Run 3 are shown in *Fig. 3*.

Table 3: Enzymes from reference fermentation

REFERENCE	Ferm 1.	Ferm 2.
C, protein (mg/ml)	1,16	1.62
PDOR (U/ml enzyme sol.)	0.25	0.26
GDH (U/ml enzyme sol.)	0.33	0.026
GDHt (U/ml enzyme sol.)	0.63	0.31

sol.: solution

PDOR activity was 10-times higher than the reference (2.64 U·ml^{-1} enzyme solution; and 0.25–0.26 U·ml^{-1} enzyme solution, respectively).

GDH activity was not detected from the optimized fermentation broth (reference: 0.33 and 0.026 U·ml^{-1} enzyme solution; carbon source glucose and glycerol or only glycerol). However GDH is of commercial trade, thus it can be a supplement for the crude enzyme solution.

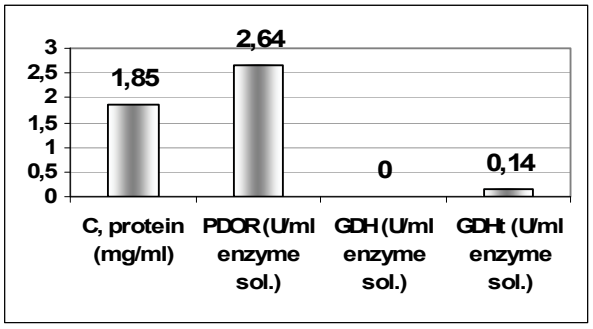

Figure 3: Enzyme activities and protein concentration from optimized fermentation

Enzymes immobilization

To carry out enzymatic bioconversions, we already used a membrane reactor system retaining the enzymes [8]. To compare it to other immobilization technics we tried a covalent immobilization method with chitosan matrix.

Chitosan was chosen as support material and crude enzyme solution from optimized fermentation broth including PDOR and GDHt enzymes supplemented with commercial GDH was applied as enzyme source. Immobilization was carried out as described in "Materials and methods".

The enzyme activity measurement method we developed for immobilized enzyme preparations was reported at Younghem2008 Conference [11].

At first NAD^+-dependent Glucose dehydrogenase (GlcDH, E. C. 1.1.1.47) from *Bacillus megaterium* was used as a test enzyme (it has commercial trade). After the process was successfully adapted, the enzyme was changed to the two key enzymes of glycerol metabolism (NAD^+-dependent PDOR and GDH). The production of NADH was monitored by the increase of absorbance at 340 nm and 25 °C. Firstly we used one bead in a cuvette (without shaking). In this case we couldn't detect enzyme activity (data shown *Fig. 4*).

As a next step we used not one but ten chitosan beads (with shaking), and measured the absorbance during the reaction in discrete points from given portion of reaction mixture (data shown *Fig. 5*). After volumetric correction the line gradient determined the NADH production rate and the immobilized enzyme activity (data shown *Fig 6*).

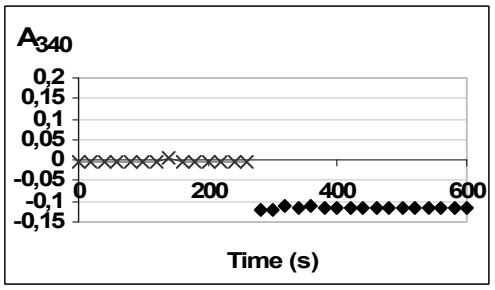

Figure 4: Immobilized enzyme activity (one bead)
- x -: background absorbance
- ♦ -: NADH absorbance

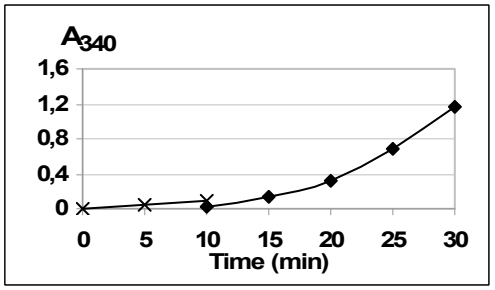

Figure 5: Immobilized enzyme activity (ten beads)
- x -: background absorbance
- ♦ -: NADH absorbance

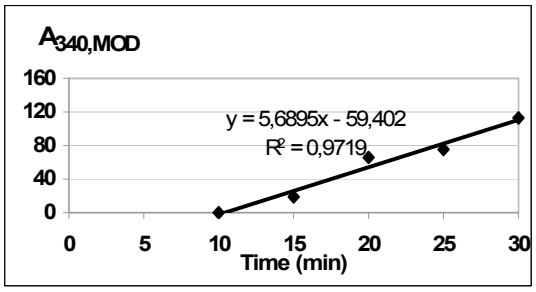

Figure 6: Immobilized enzyme activity (after volumetric correction)
- ♦ -: NADH absorbance

Soluble enzyme activity (U·ml^{-1}) was measured before and after immobilization. Enzyme activity on chitosan beads (U·gl^{-1}) was measured in the end of immobilization.

The effect of immobilization time was determined. Relative activity (%) of immobilized PDOR was measured at various immobilization time (1.5; 3; 4.5 and 24 hours; data shown *Fig. 7*). After 24 hours the relative PDOR enzyme activity decreased to 25 % of the original activity. 1.5 hours semms to be enough for immobilization.

After 1.5 hours imobilization we realised that the immobilized PDOR enzyme activity was 100 times higher and GDH enzyme activity was 160 times higher than the soluble enzyme activity (primary activity; data shown *Fig. 8*).

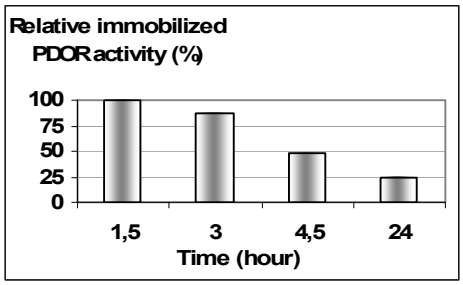

Figure 7: Effect of immobilization time

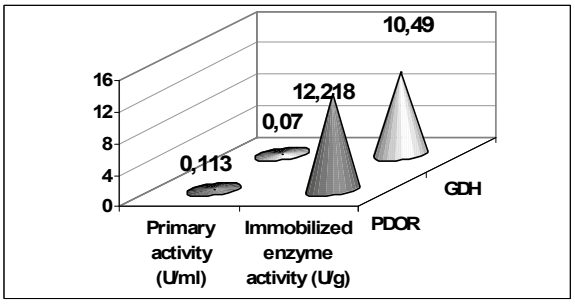

Figure 8: PDOR and GDH enzyme activity

Binding of the third enzyme, GDHt to chitosan beads applying the conditions mentioned above has not been proven, yet. Only the activity of the soluble enzyme was detectable after the immobilization procedure (data shown *Fig. 9* and *10*).This shows that in case of GDHt the applied immobilization process did not assured a certain binding, the process needed further optimization.

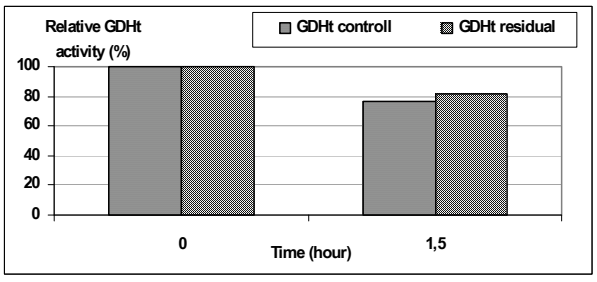

Figure 9: GDHt enzyme activity (experiment 1)

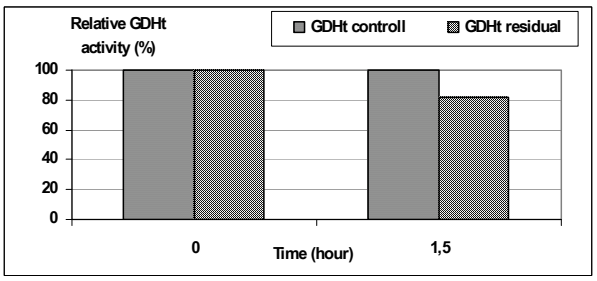

Figure 10: GDHt enzyme activity (experiment 2)

Conclusion

Using *Clostridium butyricum* as an enzyme source our preliminary results indicated that the 2xYT medium is perfect for the cultivation of the bacterium. However from economic aspects the concentrations of organic components are too high to scale-up this fermentation process. Examining the culture medium resulted in an optimal composition: 6 $g \cdot l^{-1}$ yeast extract and 2 $g \cdot l^{-1}$ bactotryptone with glycerol as carbon source and pH regulation with $CaCO_3$. To carry out enzymatic bioconversions a large amount of enzyme solution and high enzyme activity is needed. Optimized PDOR activity was 10-times higher than the reference (2.64 $U \cdot ml^{-1}$ enzyme solution; and 0.25–0.26 $U \cdot ml^{-1}$ enzyme solution, respectively).

Immobilization has been revealed as a very powerful tool to improve almost all enzyme properties. The effect of immobilization time was determined. Too long immobilization time caused significant activity lost, 1.5 hours seemed to be enough for immobilization of the PDOR enzyme. Applying the the 1.5 hour imobilization process the immobilized PDOR enzyme activity was 100 times higher and GDH enzyme activity was 160 times higher than that of the soluble enzyme. Binding the third enzyme, GDHt to chitosan beads has not been proven, yet.

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