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An increasing of the efficiency of microbiological synthesis of 1,3-propanediol from crude glycerol by the concentration of biomass

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ABSTRACT

Background: 1,3-Propanodiol (1,3-PD), is used in the production of polytrimethylene terephthalate (PTT), an aromatic polyester that exhibits high elastic recoveries. It is also employed as a supplement with low solidification properties, a solvent and a lubricant in the form of propylene glycol. 1,3-PD is effectively synthesized by a microbiological way from crude glycerol. The main problem of this technology is using a high concentration of glycerol, which is a limiting factor for bacteria cells growth (especially in batch fermentation).
Results: In this work, the influence of different glycerol concentration in batch fermentation on Clostridium butyricum DSP1 metabolism was investigated. The biomass was concentrated for two times with the use of membrane module (in case of increasing kinetic parameters). Increased optical density of bacteria cells six times increased the productivity of 1,3-PD in cultivation with 20 g/L of glycerol at the beginning of the process, and more than two times in cultivation with 60–80 g/L. Also the possibility of complete attenuation of 140 g/L of crude glycerol in the batch fermentation was investigated. During the cultivation, changes of protein profiles were analyzed. The most significant changes were observed in the cultivation in the medium supplemented with 80 g/L of glycerol. They related mainly to the DNA protein reconstructive systems, protective proteins (HSP), and also the enzymatic catalysts connected with glycerol metabolic pathway.
Conclusions: The application of filtration module in batch fermentation of crude glycerol by C. butyricum DSP1 significantly increased the productivity of the process.
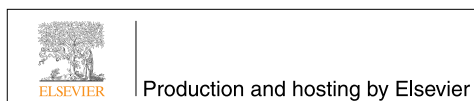
1. Introduction

The production of biofuels from renewable energy is one of the most important issues of the industrial biotechnology of the 21st century. One example of this process is the production of biodiesel from rapeseed oil. During this process, crude glycerol, as a by-product, is synthesized. There are a number of well-known methods of the application of crude glycerol, e.g. microbial utilization to 1,3-Propylene glycol (1,3-PD) using chemical synthesis of polyesters and polyurethanes [1,2,3,4]. Biotechnological production of 1,3-PD (with microorganisms) is a good alternative to a chemical way which generated huge cost and toxic by-products [5]. A very important issue is also the industrial application of crude glycerol — a by-product from biodiesel production. Microbiological synthesis of 1,3-PD is mainly carried out by bacteria from the genera Clostridium, Klebsiella, Citrobacter and Lactobacillus [3,6,7,8]. However, microbiological synthesis of 1,3-PD has some limitations, e.g. in batch and fed-batch fermentations' high concentration of glycerol increases the osmotic pressure which is a factor limiting the growth of bacterial biomass [9,10,11]. The maximum density of Clostridium butyricum cells in propanediol fermentation is 0.61–3.4 g/L (in batch process) and 4.2 g/L (in fed-batch process) and depends mostly on the concentration and purity of raw material used [7,12], while the productivity is 0.3–2.3 g/L/h in batch fermentation, 0.7–2.9 g/L/h in fed-batch fermentation, and 16.2 g/L/h in continuous process [13,14,15,16,17]. Among favorable solutions in order to improve some kinetic properties of a biotechnological way of 1,3-PD production there is biomass concentration. The advantage of this method is that it applies the process of microfiltration (MF). During MF small molecules, bacteria cells, viruses, particles of plant raw materials, and particles of fat are removed. Thus, the color of permeate can change, and its turbidity can decrease. MF results from different hydrostatic pressure between both sides of the membrane. It is commonly used in food industry, among other processes in cold sterilization of beer, wine, milk and in clarification of fruit juice. In biotechnology, it is a convenient sterilization method applied to media containing thermolabile compounds. Furthermore, filtration is commonly utilized in concentration of bacterial

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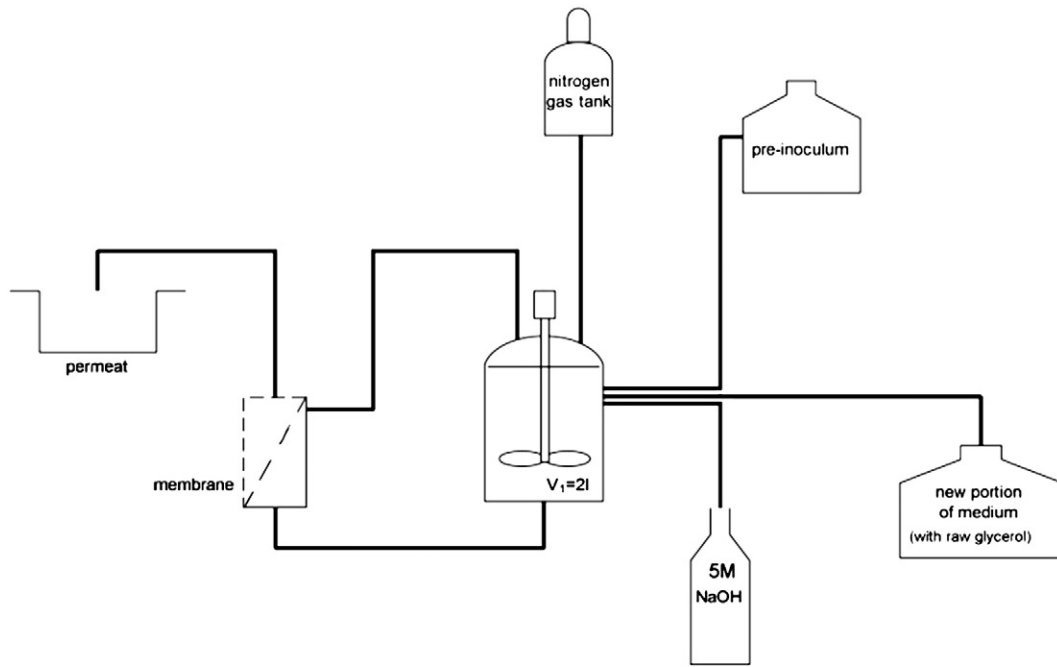ELSEVIER | Production and hosting by Elsevier

Fig. 1. The block diagram of the fermentation process with filtration module.

biomass in the production of industrially useful enzymes, therapeutic proteins, *etc.* MF is also used to concentrate algae biomass during bioethanol production [18,19].

The main aim of using filtration module in 1,3-PD from crude glycerol by microbiological way is to increase the kinetic parameters of that process and recirculation of biomass. The application of MF process in 1,3-PD production by *C. butyricum* makes it possible to concentrate biomass in closed systems which are a very important quality with respect to anaerobic microorganisms. In this work, the possibility of using MF process for biomass concentration of *C. butyricum* cells and in

the resulting process of improving kinetic parameters of 1,3-PD production was investigated.

2. Materials and methods

2.1. Microorganism

In the conversion process of crude glycerol to 1,3-PD a bacterial strain, *C. butyricum* DSP 1, was used. *C. butyricum* DSP1 was previously isolated from ruminal fluid and collected at the Department of Biotechnology and

Table 1
Experimental results of *C. butyricum* DSP 1 during batch cultivation in 2-L bioreactor, at various initial crude glycerol concentrations without biomass recycling.

Parameter/concentration of raw glycerol	20	40	60	80	100	120	140
Time of fermentation (h)	17.5	22.5	25.5	33.5	76	108	120
Max biomass, X_{max} (g/L)	0.9	1.3	1.4	1.2	0.8	0.5	0.5
Max 1,3-PD concentration, $1,3PD_{max}$ (g/L)	9.33 ± 0.12	18.83 ± 0.18	32.54 ± 0.98	37.59 ± 0.75	48.12 ± 0.22	11.22 ± 0.43	1.43 ± 0.09
1,3-PD productivity $P_{1,3-PD}$ (g/L/h)	0.53	0.83	1.28	1.13	0.63	0.1	0.01
1,3-PD yield, $Y_{1,3-PD}$ ($g_{1,3\,PD}/g_{Gly}$)	0.47	0.47	0.54	0.47	0.48	0.48	0.47
Max butyric acid concentration, But_{max} (g/L)	1.14 ± 0.08	2.23 ± 0.08	3.82 ± 0.07	4.81 ± 0.05	5.52 ± 0.06	0.02 ± 0.00	0.04 ± 0.00
Butyric acid productivity P_{But} (g/L/h)	0.34	0.27	0.29	0.32	0.21	<0.00	<0.00
Butyric acid yield, Y_{But} (g_{But}/g_{Gly})	0.06	0.05	0.07	0.06	0.05	<0.00	<0.00
Max acetic acid concentration, Ace_{max} (g/L)	0.71 ± 0.01	1.12 ± 0.03	2.2 ± 0.02	2.12 ± 0.03	2.8 ± 0.02	0.01 ± 0.00	0.02 ± 0.00
Acetic acid productivity P_{Ace} (g/L/h)	0.04	0.05	0.07	0.07	0.04	<0.00	<0.00
Acetic acid yield, Y_{Ace} (g_{Lac}/g_{Gly})	0.03	0.03	0.04	0.03	0.00	<0.00	<0.00
Max lactic acid concentration Lac_{max} (g/L)	1.04 ± 0.02	1.24 ± 0.03	2.66 ± 0.04	3.12 ± 0.04	3.36 ± 0.04	0.01 ± 0.04	0.02 ± 0.04
Lactic acid productivity P_{Lac} (g/L/h)	0.06	0.05	0.10	0.01	0.04	<0.00	<0.00
Lactic acid yield Y_{Lac} (g_{Lac}/g_{Gly})	0.05	0.03	0.04	0.04	0.03	<0.00	<0.00

Table 2

Experimental results of *C. butyricum* DSP 1 during batch cultivation in 2-L bioreactor, at various initial crude glycerol concentrations with biomass recycling.

Parameter/concentration of raw glycerol	20	40	60	80	100	120	140
Time of fermentation (h)	3.6	8	14.5	15.0	25	36	81
Max biomass, X_{max} (g/L)	2.9	3.3	3.4	3.8	3.5	3.1	2.7
Max 1,3-PD concentration, $1{,}3PD_{max}$ (g/L)	11.74 ± 0.91	22.81 ± 0.93	33.51 ± 1.01	41.22 ± 1.09	53.21 ± 1.11	59.53 ± 1.14	67.11 ± 1.19
1,3-PD productivity $P_{1,3\text{-PD}}$ (g/L/h)	3.21	2.85	2.32	2.75	2.12	1.65	0.82
1,3-PD yield, $Y_{1,3\text{-PD}}$ ($g_{1,3\,PD}/g_{Gly}$)	0.58	0.57	0.56	0.51	0.53	0.50	0.48
Max butyric acid concentration, But_{max} (g/L)	$1.2\,3 \pm 0.09$	2.17 ± 0.08	4.22 ± 0.07	4.72 ± 0.07	5.22 ± 0.07	5.77 ± 0.07	6.29 ± 0.07
Butyric acid productivity P_{But} (g/L/h)	0.34	0.27	0.29	0.32	0.21	0.16	0.08
Butyric acid yield, Y_{But} (g_{But}/g_{Gly})	0.06	0.05	0.07	0.06	0.05	0.04	0.04
Max acetic acid concentration, Ace_{max} (g/L)	0.6 ± 0.01	1.0 ± 0.02	2.0 ± 0.02	2.2 ± 0.02	2.8 ± 0.02	3.0 ± 0.02	3.2 ± 0.02
Acetic acid productivity P_{Ace} (g/L/h)	0.17	0.12	0.14	0.15	0.11	0.08	0.04
Acetic acid yield, Y_{Ace} (g_{Lac}/g_{gly})	0.03	0.02	0.03	0.03	0.03	0.02	0.02
Max lactic acid concentration, Lac_{max} (g/L)	0.94 ± 0.02	1.84 ± 0.03	2.53 ± 0.04	3.07 ± 0.04	3.53 ± 0.04	4.03 ± 0.04	4.59 ± 0.04
Lactic acid productivity P_{Lac} (g/L/h)	0.26	0.23	0.17	0.21	0.14	0.11	0.06
Lactic acid yield, Y_{Lac} (g_{Lac}/g_{Gly})	0.05	0.05	0.04	0.04	0.03	0.03	0.06

Food Microbiology, Poznan University of Life Sciences Poland, and deposited at the Polish Collection of Microorganisms PCM.

2.2. Culture medium

The Reinforced Clostridial Medium — RCM (Oxoid, UK) was used as the proliferation medium for bacteria from the genus *Clostridium*.

The composition of the fermentation medium was (per liter deionized water): 0.26 g K_2HPO_4; 0.02 g KH_2PO_4; 1.23 g $(NH_4)_2SO_4$; 0.1 g $MgSO_4 \times 7H_2O$; 0.01 g $CaCl_2 \times 2H_2O$; 0.01 g $FeCl_2 \times 7H_2O$ and 2.0 g yeast extract, and 1 mL of trace element solution SL7 [20]. The fermentation medium was supplemented with crude glycerol (Wratislavia-Bio, Wroclaw, Poland) at a concentration of $20.0–140.0 \pm 1.0$ g/L in batch fermentation. The crude glycerol composition was (w/w) 85.6% glycerol, 6% NaCl, 11.2% moisture, and pH 6.5. The media were autoclaved (121°C, 20 min).

2.3. Fermentation experiments

Fermentations were carried out in bioreactor (2 L) (Sartorius Stedim, Germany). The temperature of the process was 37°C, stirring rate was 60 rpm, pH was automatically regulated with 5 M NaOH at 7.0 ± 0.01 and with nitrogen sparged. The bioreactor was inoculated with 10% (v/v) of the pre-inoculated cultures. In cultivations with use of the membrane module, the beginning glycerol concentration was 20 g/L. In 24 h of fermentation the whole inoculated medium (2 L) was pumped on the polypropylene membrane Microdyn®MD 020 FP 2 N (Weisbaden, Germany) (20 mm × 0.2 μm) in order to separate biomass and suspended fraction (permeate). Biomass was sluiced down by a new portion of medium (1 L). Concentrations of raw materials in the new portions of medium were: 40, 60, 80, 100, and 140 g/L. The block diagram of the experiment was given in Fig. 1.
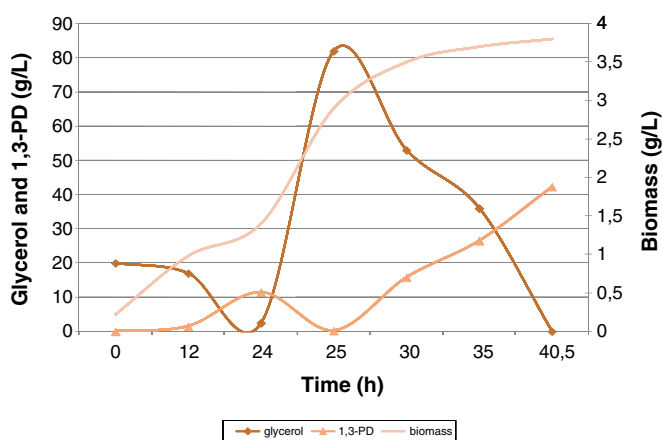
2.4. Analytical methods

1,3-PD, glycerol and organic acids were assayed by high performance liquid chromatography.

Samples for chemical analysis were first centrifuged at 10,000 *g* for 10 min at 4°C (Multifuge 3SR, Germany), filtered through a 0.22 μm membrane filter (Millex-GS, Millipore, USA), and then analyzed on an HPLC system (Agilent Technologies 1200 series).
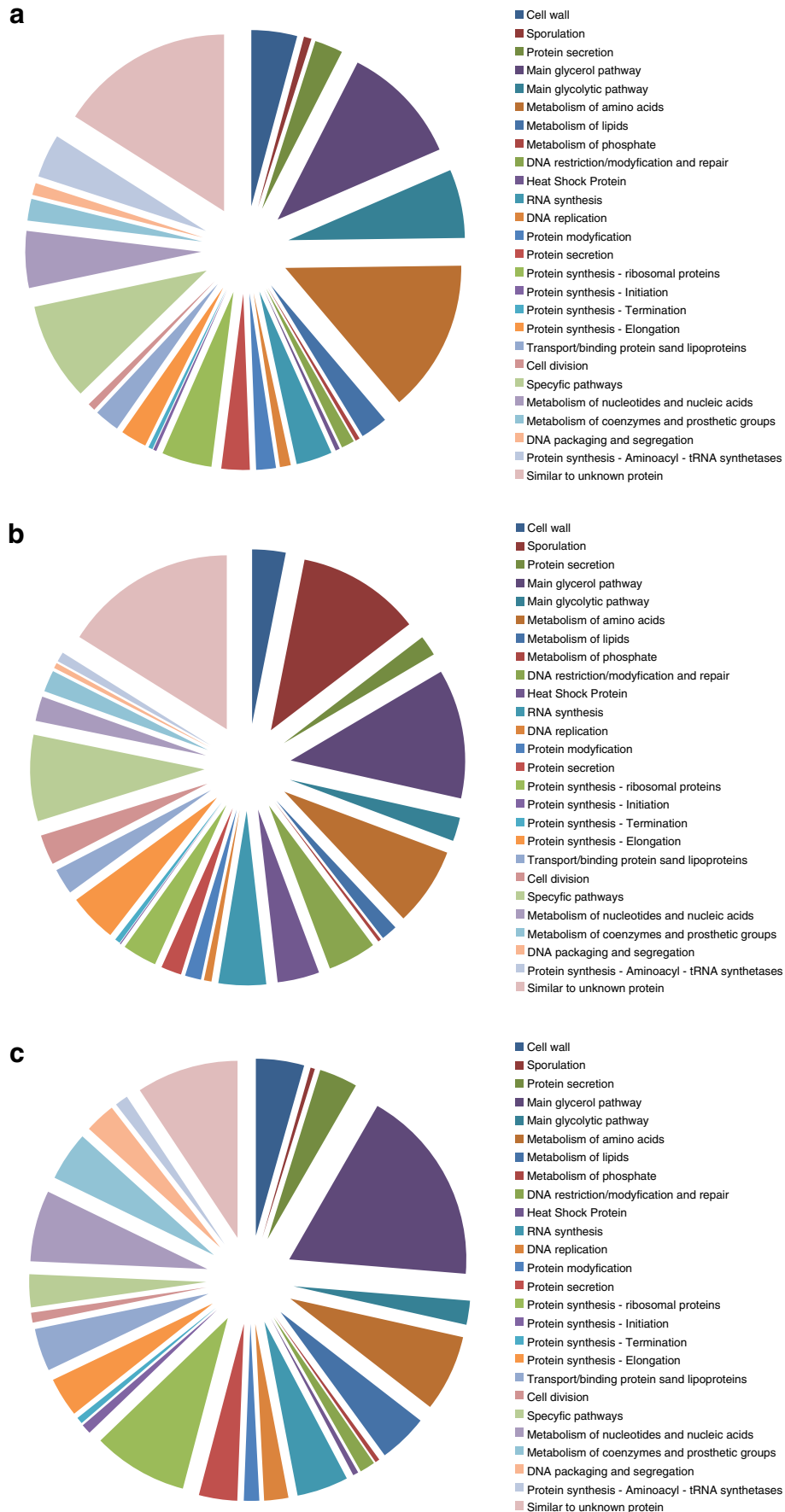
Agilent Technologies 1200 series system equipped with a refractive index detector was used. Analyses were performed isocratically at a flow rate of 0.6 mL/min on an Aminex HPX-87H 300 × 7.8 column (Bio-Rad, CA, USA) at a constant temperature of 65°C. H_2SO_4 (0.5 mN) was the mobile phase. External standards were applied for identification and quantification of peaks area. Retention times (Rt) determined for the targeted compounds for were as follows: 1,3-PD — 17.17 min; glycerol — 13.03 min; butyric acid — 20.57 min; acetic acid — 14.4 min; lactic acid — 11.19 min, and ethanol — 21.34 min.

2.5. Protein analyses

Proteins were reduced (10 mM DTT, 30 min, 56°C) and alkylated with iodoacetamide in darkness (45 min, 20°C) and digested overnight with 10 ng/uL trypsin. The resulting peptide mixtures were applied to RP-18 pre-column of the UPLC system (Waters) using water containing 0.1% FA as a mobile phase and then transferred to a nano-HPLC RP-18 column (an internal diameter 75 μM, Waters) using ACN gradient (0–35% ACN in 160 min) in the presence of 0.1% FA at a flow rate of 250 μL/min. The column outlet was coupled directly to the ion source of Orbitrap Velos mass spectrometer (Thermo). Each sample was measured in duplicate — once for protein sequencing (data-dependent MS to MS/MS switch) and once for quantitative information (MS only, sequencing disabled). The acquired MS/MS data were pre-processed with Mascot Distiller software (v. 2.3, MatrixScience) and a search was performed with the Mascot Search Engine (MatrixScience, Mascot Server 2.4) against the set of *Clostridium* protein sequences derived from Uniprot, merged with its randomized version (16,294 sequences; 5,095,802 residues). Proteins that exactly matched the same set of peptides were combined into a single cluster. The mass calibration and data filtering were carried out with MScan software. The lists of peptides that matched the acceptance criteria from the LC-MS/MS runs were

Fig. 2. Kinetics of glycerol consumption and biomass, 1,3-PD production during the growth of *C. butyricum* DSP1 on crude glycerol in batch bioreactor experiments with biomass recycling.

a

Legend (a):
- Cell wall
- Sporulation
- Protein secretion
- Main glycerol pathway
- Main glycolytic pathway
- Metabolism of amino acids
- Metabolism of lipids
- Metabolism of phosphate
- DNA restriction/modyfication and repair
- Heat Shock Protein
- RNA synthesis
- DNA replication
- Protein modyfication
- Protein secretion
- Protein synthesis - ribosomal proteins
- Protein synthesis - Initiation
- Protein synthesis - Termination
- Protein synthesis - Elongation
- Transport/binding protein sand lipoproteins
- Cell division
- Specyfic pathways
- Metabolism of nucleotides and nucleic acids
- Metabolism of coenzymes and prosthetic groups
- DNA packaging and segregation
- Protein synthesis - Aminoacyl - tRNA synthetases
- Similar to unknown protein

b

Legend (b):
- Cell wall
- Sporulation
- Protein secretion
- Main glycerol pathway
- Main glycolytic pathway
- Metabolism of amino acids
- Metabolism of lipids
- Metabolism of phosphate
- DNA restriction/modyfication and repair
- Heat Shock Protein
- RNA synthesis
- DNA replication
- Protein modyfication
- Protein secretion
- Protein synthesis - ribosomal proteins
- Protein synthesis - Initiation
- Protein synthesis - Termination
- Protein synthesis - Elongation
- Transport/binding protein sand lipoproteins
- Cell division
- Specyfic pathways
- Metabolism of nucleotides and nucleic acids
- Metabolism of coenzymes and prosthetic groups
- DNA packaging and segregation
- Protein synthesis - Aminoacyl - tRNA synthetases
- Similar to unknown protein

c

Legend (c):
- Cell wall
- Sporulation
- Protein secretion
- Main glycerol pathway
- Main glycolytic pathway
- Metabolism of amino acids
- Metabolism of lipids
- Metabolism of phosphate
- DNA restriction/modyfication and repair
- Heat Shock Protein
- RNA synthesis
- DNA replication
- Protein modyfication
- Protein secretion
- Protein synthesis - ribosomal proteins
- Protein synthesis - Initiation
- Protein synthesis - Termination
- Protein synthesis - Elongation
- Transport/binding protein sand lipoproteins
- Cell division
- Specyfic pathways
- Metabolism of nucleotides and nucleic acids
- Metabolism of coenzymes and prosthetic groups
- DNA packaging and segregation
- Protein synthesis - Aminoacyl - tRNA synthetases
- Similar to unknown protein

Fig. 3. The changes in proteins profile of *C. butyricum* DSP1 in different variants of the synthesis process 1,3-PD from the crude glycerol. Culture conditions: T = 37°C, pH 7.0, growth in a 2 L bioreactor, a) initial glycerol concentration 20 ± 1.0 g/L, without biomass recycling; b) initial glycerol concentration 120 ± 1.0 g/L, without biomass recycling; c) initial glycerol concentration 20 ± 1.0 g/L, with recycling biomass, glycerol concentration after biomass recycling 120 ± 1.0 g/L.

merged into one common list. This common list was overlaid onto 2-D heat maps generated from the LCMS profile data sets by tagging the peptide-related isotopic envelopes with corresponding peptide sequence tags on the basis of the measured/theoretical mass difference, the deviation from the predicted elution time, and the match between the theoretical and observed isotopic envelopes. The abundance of each peptide was determined as the height of a 2-D fit to the monoisotopic peak of the tagged isotopic envelope. Quantitative values were normalized with LOWESS, proteins with more than 80% common peptides were clustered and only peptides unique for the cluster were used for statistical analysis. Only proteins with value below 0.05 or those present in only one of two compared analytical groups were taken into consideration during further analysis. The protein concentration was measured by the Bradford's method [21].

3. Results

3.1. Influence of different concentration of crude glycerol on kinetic parameters of 1,3-PD production by C. butyricum DSP1

In 1,3-PD production by biotechnological way, raw material and product both may limit the efficiency of metabolite production [10,11,12,22]. Glycerol and 1,3-PD have high osmotic pressure, so they can cause damage to bacteria cells. Thus, a very important step in selection of new isolates to this metabolite production is to investigate the boundary concentration which does not negatively influence bacteria cells and the final concentration of products. In the first stage, the influence of different concentration of crude glycerol on kinetic parameters of 1,3-PD production by *C. butyricum* DSP1 in batch fermentation was investigated (Table 1). The aim of this part of the work was to determine the highest concentration of crude glycerol (at the beginning of fermentation) which does not inhibit bacterial growth and 1,3-PD synthesis.

The highest concentration of crude glycerol which was not completely fermented by *C. butyricum* DSP1 was 80 g/L in batch fermentation. The efficiency of that process was 0.47 g/L and productivity 1.13 g/L/h. It is an important issue that native *C. butyricum* DSP1 has low biomass concentration during fermentation, *i.e.* not exceeding 1.4 g/L (Table 1). This experiment demonstrated that concentration of crude glycerol more than 100 g/L does inhibit microorganism metabolism. In effect, microorganisms cannot utilize the whole amount of carbon from the fermentation medium.

3.2. Influence of biomass concentration on fermentation kinetic parameters

One way to improve kinetic parameters of fermentation process is increasing microorganisms' concentration in the fermentation medium. In the next stage of this work the density of bacterial biomass was increased two times using membrane module. The influence of concentrated biomass on the efficiency of 1,3-PD was investigated in fermentation medium with different crude glycerol concentrations (in the range of 20 to 140 g/L). During fermentation, metabolite production, the density of the cells, and protein profiles were analyzed. Results of these experiments are presented in Table 2. It was found out that the use of biomass with two times concentrated bacteria exerted the main kinetic properties of that process (a significant influence of the productivity was observed). Also, in new fermentative medium, the lack of an adaptive phase was observed (in all glycerol concentration) (Fig. 2), which also increased the efficiency of 1,3-PD synthesis. Additionally, concentration of bacteria cells increased efficiency of crude glycerol utilization. Generally, the efficiency of 1,3-PD production was ca. 10% higher in fermentation with concentrated biomass (in batch process) (67.11 g/L). A higher level of utilized crude glycerol (140 g/L) was observed (Table 2).

Kinetics of glycerol utilization, metabolite production and biomass increase during fermentation of crude glycerol (80 g/L) using membrane module and concentrated biomass is presented in Fig. 2. This process had

favorable parameters — high productivity (2.71 g/L/h), high biomass concentration, and high amount of metabolite were observed. However, there was no significant difference between the level of other metabolites (lactic, acetic, and butyric acids) in fermentation with or without biomass concentration. The general conclusion about by-products was thus that the higher glycerol concentration, the higher by-products synthesis.

3.3. Proteomic analysis of C. butyricum from glycerol fermentation

In the next stage of this work, the protein profile of *C. butyricum* during synthesis of 1,3-PD in fermentation with and without concentration was analyzed. The concentration at the beginning of cultivation was 120 g/L. The fermentation with 20 g/L of glycerol at the beginning was a control probe (without concentration of biomass). Fig. 3 presents percentage participation of all identified intracellular proteins in all tested options. In control probe mostly factors which take part in proteins synthesis and secretion, as well as proteins taking part in glycerol and glycolysis pathways were detected. In fermentation with 120 g/L of glycerol and concentration of biomass equal to 0.9 g/L the highest percentage participation of chaperon proteins and proteins repair DNA (in comparison to other fermentation options) was observed. It indicated that complicated systems of DNA repair, as well as molecules protecting functional proteins were activated. Probably, expression of genes encoding proteins taking part in glycerol conversion is attenuated (Fig. 3b). In fermentation with adaptation of microorganism in medium with 20 g/L of glycerol and concentrated biomass before inoculation into medium with high osmotic pressure (120 g/L of crude glycerol) another protein profile was observed than in the option without biomass concentration (Fig. 3c). The percentage participation of proteins taking part in glycerol and amino acids pathway was the highest in comparison to other fermentation options. The level of proteins repair DNA and taking part in cells sporulation was on the comparable level than in fermentation with optimal glycerol concentration.

4. Discussion

The main parameter which influences kinetic parameters of 1,3-PD production of *C. butyricum* is low biomass concentration. Generally, it is a problem of batch fermentation and glycerol as the only carbon source [4,7,16,20,23]. In literature, there are descriptions of many options of cultivation in which the main aim is to increase the concentration of bacterial biomass. In case of 1,3-PD production, one of these methods is fed-batch fermentation, continuous fermentation with cells recirculation, batch and continuous fermentation with immobilized cells, and multi-stage fermentation [9,15,20,24,25,26]. The main novelty of the work described by the present authors was application of microfiltration membrane in batch fermentation process. This membrane was applied in order to separate bacterial biomass in anaerobic conditions and to use them anew. In the first step of this task the maximal concentration of glycerol (which may be completely utilized by microorganisms) was determined. The level of glycerol tolerance is strictly dependent on bacterial strains. In the literature data batch fermentations with 10–50 g/L are typically described [7,10,12,26,27,28]. Obtained results show that high tolerance of *C. butyricum* DSP1 towards osmotic pressure enables the use of high glycerol concentration without significant negative impact on kinetic parameters of the fermentation.

In batch fermentation with MF strain *C. butyricum* DSP 1 was able to ferment 140 g/L of glycerol and synthesize 67 g/L of 1,3-PD which is comparable with the results obtained in fed-batch processes by some scientists [11,16]. However, the main disadvantages of fed-batch fermentation are not-completed glycerol utilization and long duration of the process. Additionally, in fed-batch cultivation, the fermentative medium is diluting and finally the main product of glycerol metabolism is also diluting. For example, Hirschmann et al. [9] in repeated batch fermentation obtained 87.7 g/L of glycerol (productivity 1.9 g/L/h).

This process lasted 46 h. In our work, complete crude glycerol was fermented 1.4 times faster. 80 g/L was utilized during 15 h, so productivity was 2.75 g/L/h. This result is comparable with productivity obtained in continuous fermentations [12]. Although, the main aim of continuous process is not the production of high amount of metabolites [12]. Papanikolaou et al. [20] obtained 48 g/L of 1,3-PD in continuous fermentation (productivity 5.5 g/L/h). Chateau et al. [29] in their patent described the process of continuous fermentation results with the efficiency equal to 0.53 g/g of glycerol, final concentration of 1,3-PD on the level of 53 g/L and the productivity equal to 2.87 g/L/h. Very high productivity (16.9 g/L/h) was obtained by Suratago and Nootong [17] in continuous moving bed fermentation by *C. butyricum* DSM 5431. However, the final concentration of 1,3-PD was only 33.8 g/L. The application of membrane module is described in other works, such as by Ennis and Maddox [30] and Tashiro et al. [31]. The aim of MF was to improve the kinetic parameters of ABE fermentation carried out by *Clostridium saccharoperbutylacetonicum* and *Clostridium sacarobutylicum*. Glycerol and galactose were used as raw materials, the productivity obtained in these processes was, respectively, 11.0 and 4.06 g/L/h. Researches on the conversion of crude glycerol to 1,3-PD by physical methods, such as ultrasounds were carried out by Khanna et al. [25,26,32]. Also other methods increasing 1,3-PD production and glycerol consumption were tested by scientists. Application of ultrasounds in glycerol conversion using immobilized *C. pasteurianum* cells increased glycerol consumption was researched by Khanna et al. [3]. The authors stated that ultrasonication of the fermentation mixture leading to increase in the effect of substrate-enzyme complex and decrease substrate inhibition for 1,3-PD dehydrogenase, which causes propanediol pathway is preferential. In other work the same authors tested the influence of the temperature on the efficiency of 1,3-PD production by *C. pasteurianum* [26]. The temperature of 37°C was optimal for butanol production, and 30°C was optimal for 1,3-PD and ethanol synthesis from crude glycerol. The authors compared these observations with optimal temperatures for enzymes taking part in metabolic pathways.

The critical concentrations of crude glycerol in this work were 120 and 140 g/L (Table 1 and Table 2). These concentrations caused weak 1,3-PD production, decreased a number of microorganisms, as well as their vitality and metabolic activity, and also protein profile changed. A very important issue is the possibility to analyze the protein profile of the cells and changes during fermentation process [33,34]. In our work, more significant changes in protein profile were observed in fermentations with high glycerol concentration (Fig. 3b and c). In fermentation with 120 g/L of glycerol (without biomass concentration) the level of protective proteins (mainly HSP) and transcription factor of sporulation process (SpoOA) increased, simultaneously the number of enzymatic enzymes taking part in glycerol metabolism decreased. It indicated that mechanisms responsible for cells protection were activated [34,35]. The number of enzymatic proteins taking part in glycerol metabolism in fermentation with 120 g/L of glycerol was comparable with fermentation in which glycerol concentration was six times lower. Nevertheless, the number of protective proteins was sign6ificantly higher in fermentation with higher glycerol concentration (Fig. 3b). The main advantages of biomass concentration in glycerol fermentation by *C. butyricum* DSP1 include the utilization of high concentration of raw material and the lack of biomass multiplying step. However, the time of utilization of glycerol was longer and productivity decreased to the level of 0.82 g/L/h than in the fermentation without biomass concentration. The reason why the fact is probably that crude glycerol contains some impurities. In cultivation with concentrated biomass, the level of glycerol is high so the level of impurities is higher than in classic fermentation process [36,37,38]. It influenced on activation of protection proteins and inactivation of enzymatic proteins which convert glycerol to 1,3-PD (in case of saving energy). Additionally, decreased productivity (Table 2) is probably connected not only with high raw material concentration, but also with other stress factors,

such as toxic by-products of glycerol pathway (*e.g.*, organic acids and ethanol) [38,39]. One way to solve this problem is to remove organic acids. However, methods of this process have a lot of disadvantages, *e.g.* it must be done as a separate step (not during fermentation) and is expensive [12]. Thus, organic acids are also limiting factors of utilization of high concentration of glycerol by microorganisms [39].

The application of fil module in batch fermentation of crude glycerol by *C. butyricum* DSP1 significantly increased the productivity of the process. Moreover, complete glycerol was utilized during such fermentation. The analyses of proteomic profile during bacteria fermentation demonstrated that in bacteria cells there are some mechanisms which protect metabolic pathways and prevent cell from dying. However, these mechanisms (activated, for example, in osmotic stress) weaken kinetic parameters of the main process — synthesis of metabolites.

Conflict of interest

The Authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Acknowledgments

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