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The effect of high concentrations of glycerol on the growth, metabolism and adaptation capacity of *Clostridium butyricum* DSP1

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ABSTRACT

Background: The production of biofuels from renewable energy sources is one of the most important issues in biotechnology today. The process is known to generate various by-products, for example glycerol that is obtained in the making of biodiesel from rapeseed oil. Crude glycerol may be utilized in many ways, including microbial conversion to 1,3-propanediol. The main drawback of that technology is the use of high concentrations of glycerol, which inhibits the growth of bacterial cells.

Results: This study investigated the impact of crude glycerol on *Clostridium butyricum* DSP1 and its ability to adapt to an environment of high osmotic pressure. It was found that a crude glycerol concentration of up to 70 g/L did not have an inhibitory effect on *C. butyricum* DSP1. Adaptation procedures involving the passage of metabolically active biomass from a fermentation medium with a lower concentration of crude glycerol to one with a greater substrate concentration allowed breaking the barrier of high osmotic pressure (150 g/L crude glycerol) and receiving a 1,3-PD concentration of 74 g/L in a batch culture operation. The work looked into intracellular modifications shown by proteomic profiling in order to explain the mechanisms underlying the response and adaptation of bacterial cells exposed to unfavorable environmental conditions.

Conclusions: This study of the effect of glycerol on the growth and metabolism of *C. butyricum* DSP1 demonstrated that the maximum substrate concentrations that do not inhibit the metabolic activity of bacterial cells are 90 g/L and 70 g/L for pure and crude glycerol, respectively.

1. Introduction

One of the most pressing problems of industrial biotechnology today is a need to select microorganisms robustly resistant to adverse environmental conditions. The metabolic and molecular mechanisms of cellular response to various stress factors have been dealt with in many publications concerned predominantly with laboratory research rather than studies of industry-scale fermentation. It is vital, however, to understand the dependence of microbial behavior on conditions that occur during industrial processes in order to ensure optimal selection of strains and efficient management of mass-scale fermentation [1]. An important biotechnological issue is the inhibition of bioconversion that may arise from considerable concentrations of substrates or products (high osmotic pressure in the fermentation medium) and/or the presence of toxic substances (metabolites and/or substrate impurities) [2,3]. Bacterial growth inhibition also affects microorganisms of the genus *Clostridium*. The non-pathogenic clostridia have a great potential for industrial applications [4]. These bacteria ferment organic compounds such as carbohydrates and release large amounts of CO_2 and H_2 as well as organic acids (*e.g.* butyric, lactic, acetic, and succinic acids) and solvents such as butanol, acetone, and isopropanol (solventogenic clostridia) [4,5]. Among these substances, 1,3-propanediol (1,3-PD) is of industrial interest as a monomer for light-insensitive plastics. 1,3-PD is an industrially valuable chemical intermediate with potential uses in the production of polymers (*e.g.* polyesters, polyethers, polyurethanes), cosmetics, foods, lubricants, medicines, and as an intermediate for the synthesis of heterocyclic compounds such as indole and quinolines [4,5,6,7,8]. 1,3-PD may be produced chemically or microbiologically [9,10,11,12]. At present chemical methods are being replaced by microbiological technologies. 1,3-PD is effectively synthesized microbiologically from crude glycerol generated during biodiesel production [3,8]. In the microbiological conversion of glycerol to 1,3-PD the most common are *Clostridium* spp., *Klebsiella* spp., *Citrobacter* spp., *Lactobacillus* spp. and *Hafnia* spp. [8,13,14,15]. The main factors responsible for inhibiting that process include a high substrate concentration (pure or crude glycerol), the accumulation of toxic products such as 1,3-PD, and the presence of organic acids. This study focused on defining the maximum concentration of glycerol that allows bacterial growth and efficient

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synthesis of 1,3-PD. Additionally, an attempt was made to adapt *Clostridium butyricum* DSP1 to high concentrations of crude glycerol.

2. Materials and methods

2.1. Microorganism

The strain used in the process of converting crude glycerol to 1,3-PD was *C. butyricum* DSP1 [16]. It was previously isolated from ruminal fluid and put in the collection of the Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Poland, as well as deposited at the Polish Collection of Microorganisms (PCM).

2.2. Culture medium

The strain was maintained in Reinforced Clostridial Medium (RCM, Oxoid, UK) in serum bottles at 4°C. Pre-cultures of pure culture inoculum were cultivated in Hungate test tubes in an appropriate cultivation medium (37°C, 18 h). *Clostridium* bacteria were cultured in a chamber for cultivation of anaerobic microorganisms (Whitley MG500, Don Whitley Scientific, Shipley, United Kingdom), without pH regulation or stirring.

2.3. Fermentation medium

The first fermentation medium consisted of (per 1 L of 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 yeast extract. The concentration of the yeast extract in the fermentation medium was increased as the glycerol concentration rose (Table 1). The fermentation medium was supplemented with pure (Avantor Performance Materials Poland S.A, Gliwice) or crude glycerol (Wratislavia-Bio, Wroclaw, Poland) at a concentration of 50.0–170.0 ± 1.0 g/L. The crude glycerol contained (v/v) 72% glycerol, 12% NaCl, and 13.6% water, and had a pH of 6.5. The media were autoclaved at 121°C for 20 min.

2.4. Fermentation experiments

All experiments were performed in a 6.6 L bioreactor (Sartorius Stedim, Germany) with a working volume of 2.0 L. The bioreactor was equipped with controls for the temperature, pH, agitation speed and aeration rate. The pH was controlled at 7.0 by automatic addition of 1 M NaOH and all fermentation experiments were carried out at 37°C. Anaerobic conditions were sustained by continuous nitrogen sparging at a flow rate of 0.1 vvm. During the experiments aimed at examining the influence of high glycerol concentrations on *C. butyricum* DSP1 the biomass was passaged from the culture medium to production media where pure or crude glycerol concentrations were 50, 70, 90, 110, 130, 150, and 170 g/L. An evaluation of the adaptation ability of *C. butyricum* DSP1 was performed using only crude glycerol and comprised several stages of which the first one involved a batch culture with a substrate concentration of 70 g/L. The moment that biomass accumulation reached a plateau, an amount of the bioreactor's contents equal to 10% of its working capacity was pumped into another one filled with a production medium containing 90 g/L crude glycerol. The next passages were carried out to production media with glycerol concentrations of 110, 130, 150, and 170 g/L in a similar manner.

Table 1
The concentration of yeast extract depending on the applied concentration of glycerol.

Concentration of glycerol (g/L)	Concentration of yeast extract (g/L)
50	2
70	4
90	6
110	8
130	10
150	12
170	14

2.5. Analytical methods

The 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).

An 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 columns (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 peak areas. Retention times (Rt) determined for the target compounds were: 1,3-PD — 17.17 min; glycerol — 13.03 min; butyric acid — 20.57 min; acetic acid — 14.4 min and lactic acid — 11.19 min.

The cell concentration (g/L) was determined using a linear equation derived from the relationship of cell dry weight (90°C until constant weight) and optical density (OD) at 600 nm (Analytik Jena Specord 50).

2.6. 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/μL trypsin. The resulting peptide mixtures were applied to the RP-18 pre-column of a UPLC system (Waters) using water containing 0.1% FA as a mobile phase and then transferred to a nano-HPLC RP-18 column (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 mL/min. The column outlet was coupled directly to the ion source of an 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 (16294 sequences; 5095802 residues).

Those proteins that exactly matched the same set of peptides were combined into a single cluster. 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 merged into one common list. This was overlaid onto 2-D heat maps generated from the LC–MS profile datasets 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 the peptides unique for the cluster were used for statistical analysis. Only proteins with q-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 Bradford's method [17].

3. Results

3.1. The effect of high concentrations of pure and crude glycerol on the growth and metabolism of C. butyricum DSP1

This part of the study involved tests designed to evaluate the resistance of *C. butyricum* DSP1 to various concentrations of pure and crude glycerol within a range of 50–170 g/L. It was found that although the growth of *C. butyricum* DSP1 bacteria and the synthesis of 1,3-PD were still possible at a pure glycerol concentration of 110 g/L, complete substrate utilization occurred in the range of 50–90 g/L. The concentration of pure glycerol at which the 1,3-PD yield reached a maximum of 0.56 g/g and the productivity stood at 1.13 g/L/h was 70 g/L.

The highest concentration of 1,3-PD, 48.3 g/L, was obtained for the initial substrate concentration of 90 g/L, though the productivity declined markedly to 0.78 g/L/h. The highest value of the initial substrate concentration allowing for a maximum of biomass aggregation and a satisfying level of 1,3-PD yield was further reduced after using crude glycerol as a carbon source and dropped to 50 g/L. Under those conditions a slight accumulation of the biomass still progressed at a crude glycerol concentration not exceeding 110 g/L whereas complete substrate utilization was observed for concentrations of 50 and 70 g/L. A significant fall in the productivity was registered as increasing initial substrate concentrations were applied. At the initial concentration of crude glycerol of 110 g/L the productivity was 0.22 g/L/h, 1.5-fold lower than that for pure glycerol of the same concentration (Table 2). The 1,3-PD yield also decreased compared to the synthesis from the pure substrate. The profile of metabolites other than 1,3-PD changed as well. Due to the fact that the use of rising concentrations of the crude glycerol caused a greater production of lactic acid, the selectivity of synthesizing particular metabolites, especially 1,3-PD, was considered to be an important kinetic parameter. The selectivity of 1,3-PD synthesis expressed as percentage was obtained by dividing the 1,3-PD concentration by the sum of the concentrations of all liquid metabolites, and multiplying by 100%. Thus, the selectivities of 1,3-PD synthesis from 50 g/L and 110 g/L crude glycerol were 74% and 63%, respectively. Biomass accumulation was stopped in the experiments with high concentrations of pure as well as crude glycerol. A 130 g/L concentration of crude glycerol prevented bacterial growth and even resulted in autolysis of the existing cells supplied with the culture medium, which was reflected by a sharp increase in the pH.

Table 2
Effect of initial glycerol concentration on growth and kinetic parameters during batch fermentation of *C. butyricum* DSP 1.

C_{Gly} (g/L)	$C_{1,3\text{-PD}}$ (g/L)	$Y_{1,3\text{-PD}}$ (g/g)	X (g/L)	Q (g/L/h)	$S_{1,3\text{-PD}}$ (%)
Pure					
50	27.5 ± 0.9	0.55	1.2 ± 0.1	1.18	82
70	38.5 ± 1.0	0.56	1.3 ± 0.2	1.13	84
90	48.3 ± 1.1	0.54	0.9 ± 0.1	0.78	73
110	36.1 ± 1.1	0.51	0.9 ± 0.1	0.31	68
130	25.3 ± 0.8	0.48	0.7 ± 0.1	0.21	65
150	20.3 ± 0.6	0.42	0.5 ± 0.0	0.23	62
170	0.3 ± 0.0	–	0.2 ± 0.0	–	–
Crude					
50	26.2 ± 0.9	0.52	1.3 ± 0.3	0.94	74
70	35.8 ± 0.9	0.51	1.2 ± 0.1	0.86	77
90	28.3 ± 0.8	0.47	0.8 ± 0.1	0.62	64
110	30.8 ± 0.8	0.48	0.5 ± 0.0	0.22	63
130	0.5 ± 0.0	–	0.2 ± 0.0	–	–
150	0.0 ± 0.0	–	0.2 ± 0.0	–	–
170	0.0 ± 0.0	–	0.1 ± 0.0	–	–

C_{Gly}: concentration of glycerol in fermentation medium (gram per liter); $C_{1,3\text{-PD0}}$: produced 1,3-propanediol (gram per liter); $Y_{1,3\text{-PD}}$: yield of 1,3-propanediol produced per glycerol consumed (gram per gram); X: biomass (gram per liter); Q: 1,3-propanediol productivity (gram per liter per h); $S_{1,3\text{-PD}}$: selectivity of 1,3-PD synthesis (percentage); dividing 1,3-PD concentration by sum of concentrations of all liquid metabolites, and multiplying by 100%.

3.2. The adaptation of C. butyricum DSP1 to large concentrations of crude glycerol

In this part of the study an attempt was made to adapt *C. butyricum* DSP1 bacteria to both osmotic (large substrate concentrations) and toxic (crude glycerol) stress conditions. Five batches of metabolically active biomass were passaged from the first fermentation medium (70 g/L crude glycerol) to the next ones containing successively greater glycerol concentrations (90, 110, 130, 150, 170 g/L) (Table 3).

It was observed that the kinetic parameters of 1,3-PD fermentation improved significantly as compared to the experiment results shown in Table 2. Complete substrate utilization occurred when the concentrations of glycerol in the media were 90, 110, 130, and 150 g/L, corresponding to noteworthy productivity values of 1.01, 1.03, 1.09, and 0.74 g/L/h, respectively. The metabolite profile typified a proper process of microbiological glycerol conversion by *C. butyricum* DSP1 and the 1,3-PD synthesis selectivity ranged from 64 to 75% (Table 3). Microscopic imaging did not indicate any concern-raising morphological cell alterations (Fig. 1a). The crude glycerol concentration at which the 1,3-PD concentration reached a maximum of 74.2 g/L was 150 g/L. The process was inhibited following the passage of the biomass to the medium containing 170 g/L glycerol. Despite the availability of nutrients the productivity fell substantially to 0.31 g/L/h. Substrate utilization did not exceed 60%. Biomass accumulation reached 50% of the highest level obtained in the stage with 150 g/L crude glycerol. A marked increase in the concentration of lactic acid caused a drop in the yield of the main metabolite to 0.44 g/g. In addition, elongated forms were noticed under a microscope that appeared to be conglomerates of bacterial cells (Fig. 1b).

In order to investigate changes in the metabolism of cells exposed to various environmental stressors (high osmotic pressure, increasing concentration of toxic agents, stirring), the level of intracellular proteins in *C. butyricum* DSP1 bacteria was analyzed. The results of proteomic analysis collected in Fig. 2 represent the physiologic status of the cells during 1,3-PD synthesis at two stages using diametrically different substrate concentrations. Fig. 2a shows the analytical results for the stage with the first passage of the biomass to the fermentation medium containing 90 g/L glycerol. Changes in the levels of cell stress response markers selected for analysis observed in the stage where the fermentation was inhibited and the substrate concentration was 170 g/L are presented in Fig. 2b. The markers of interest were the proteins HSP20, HSP60, HSP70, the sporulation-related transcription factor Spo0A as well as two enzymatic proteins connected with the pathway of 1,3-PD synthesis followed by *C. butyricum*: glycerol dehydratase and 1,3-PD dehydrogenase. The analysis demonstrated considerable alterations to the proteomic profile of *C. butyricum* DSP1.

Table 3
Kinetic parameters of fermentation during adaptation to large concentrations of crude glycerol of *C. butyricum* DSP1.

Number of passage	C_{Gly} (g/L)	$C_{1,3\text{-PD}}$ (g/L)	$Y_{1,3\text{-PD}}$ (g/g)	X (g/L)	Q (g/L/h)	$S_{1,3\text{-PD}}$ (%)
0	70	34.9 ± 1.16	0.50	1.2 ± 0.1	0.86	73
1	90	45.6 ± 1.53	0.51	1.1 ± 0.1	1.01	75
2	110	55.6 ± 1.68	0.50	1.1 ± 0.1	1.03	73
3	130	64.8 ± 1.58	0.49	0.9 ± 0.1	1.09	72
4	150	74.2 ± 1.32	0.49	0.8 ± 0.1	0.74	72
5	170	43.1 ± 1.12	0.44	0.4 ± 0.1	0.31	64

C_{Gly}: concentration of glycerol in fermentation medium (gram per liter); $C_{1,3\text{-PD}}$: produced 1,3-propanediol (gram per liter); $Y_{1,3\text{-PD}}$: yield of 1,3-propanediol produced per glycerol consumed (gram per gram); X: biomass (gram per liter); Q: 1,3-propanediol productivity (gram per liter per hour) $S_{1,3\text{-PD}}$: selectivity of 1,3-PD synthesis (percentage); dividing 1,3-PD concentration by sum of concentrations of all liquid metabolites, and multiplying by 100%.

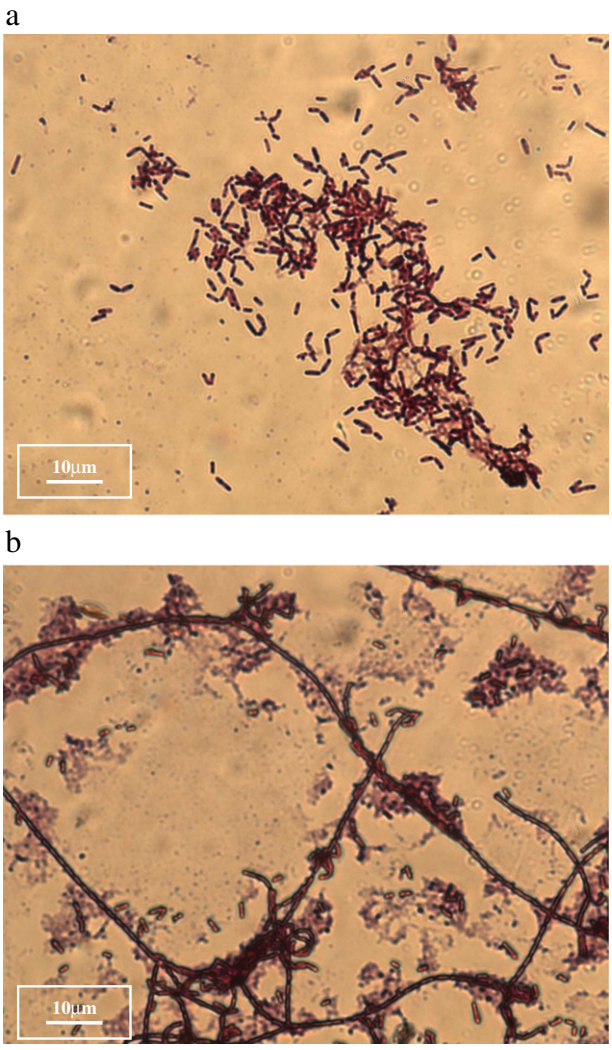

Fig. 1. Environmental impact on morphology of *C. butyricum* DSP1 (a) neutral conditions and (b) stress conditions. Neutral conditions: initial glycerol concentration 70 ± 1.0 g/L, T = 37°C, pH 7.0, growth in 2 L bioreactor. Stress conditions: initial glycerol concentration 170 ± 1.0 g/L, T = 37°C, pH 7.0, growth in 2 L bioreactor.

High levels of the HSP proteins (particularly the HSP60) and the Spo0A factor registered in the stage with 170 g/L glycerol (Fig. 2b) indicated activation of cell defense mechanisms, which was not observed when bioconversion was not disturbed (Fig. 2a). Moreover, the process of autolysis (typically marked by a surge in the pH of the fermentation medium) was not triggered in either of the stages. The level of enzymatic proteins strictly associated with the pathway of metabolic conversion of glycerol was greater in the first stage (Fig. 2a) than in the second stage (Fig. 2b) suggesting an inhibiting impact of stress conditions on their synthesis.

4. Discussion

The majority of technological processes using microorganisms proceed in conditions generating various environmental stressors that negatively affect their growth and metabolism [2,5]. One of them is a high substrate concentration, which is beneficial only in economic terms as it affords high product concentrations, thus reducing the cost of product separation and the number of fermentation cycles required. Therefore, stress factors are often applied in selection tests used in the search for new microorganisms with biotechnological potential allowing elimination of stress-prone strains at the isolation stage [18,19]. In this study an attempt was made to define the maximum concentrations of a pure and a crude glycerol allowing the growth and effective metabolism of *C. butyricum* DSP1. Since glycerol is an osmotically active substance with a significant influence on the osmotic potential of a fermentation medium, it may limit the production capacity of microorganisms [2]. It was observed that the growth and metabolism of *C. butyricum* DSP1 were not inhibited until the concentration of pure glycerol in the fermentation medium exceeded 90 g/L. However, industrial biotechnology has a particularly important task in selecting microbial strains able to metabolize waste materials, including crude glycerol [20,21,22]. The use of substrates originating as waste generates stress factors resulting from the accumulation of toxic agents in the fermentation medium, not to mention the problem of varying substrate quality even within the same operation. Many authors studying the conversion of crude glycerol to 1,3-PD have reported deterioration of kinetic parameters, especially final product concentration, as compared to pure glycerol [14,23,24]. Similar findings were made in this work (Table 2). Interestingly, a decrease was observed in the productivity and selectivity of 1,3-PD synthesis for high concentrations of both pure and crude glycerol, probably due to an increased synthesis of lactic acid by the bacteria under stress conditions. An increased content of lactic acid indicates that the process is blocked probably due to substrate excess, a high concentration of toxic carbon monoxide or stoppage at the stage of pyruvate generation. However, it should not be assumed that this tendency is always observed for the synthesis of 1,3-PD and other metabolites [7,25,26]. The maximum concentrations of a pure and a crude glycerol determined in the present study that did not inhibit *C. butyricum* DSP1 were 90 and 70 g/L, respectively, which is below the levels reported by Ringel et al. [19]. The differences between results of experiments with *C. butyricum* AKR102a and *C. butyricum* DSP1 were probably mainly due to their varied origin, including different selection factors used in the screening, and differences in the composition of the carbon-providing substrate [16,19]. Considering the unsatisfying parameters received with *C. butyricum* DSP1, in order to adapt the bacteria to conditions caused by using crude glycerol at a high concentration, batches of metabolically active biomass were passaged to the fermentation media containing successively greater substrate concentrations. This enabled a complete utilization of 150 g/L glycerol and provided a 1,3-PD concentration of 74.2 g/L, which was only 5.9 g/L less than the highest value of 80.1 g/L reported previously by Hirschmann et al. [27]. In addition, proteomic analysis conducted in this study gave an insight into modifications within *C. butyricum* DSP1 bacteria cells at the molecular level. Since changes in the levels of some intracellular proteins reflect the physiologic status of cells, it may be possible to obtain a detailed profile of a strain with potential for industrial application with emphasis on its metabolic capacity [28]. Heat shock proteins, similar to the transcription factor Spo0A, are markers for the response of *C. butyricum* to adverse environmental conditions [28,29]. An increase in their levels is a signal that the cell defense mechanism is active and that the metabolic processes are redirected toward protecting the cells from the harmful action of the environment. Consequently, an intensified synthesis of proteins that do not normally participate in the main metabolic activity slows or stops the entire process. A rise in the HSP60, a protein also known as GroEL, at the stage when the crude glycerol concentration was 170 g/L indicated the efforts of the cells to defend against the high osmotic potential in the early hours of the process. The literature points to HSP60 as a protein associated with the response of the genus *Clostridium* to osmotic, toxic and temperature stresses [29,30]. During that period biomass accumulation was not observed, which pointed to the interruption of some life-sustaining biochemical reactions, probably to save metabolic energy. The level of enzymatic proteins associated with the metabolism of glycerol continued to grow, though not as markedly as when the crude glycerol concentration was 90 g/L. The fact that it still exceeded the initial value proved the ability of the microorganisms to metabolize the substrate at such an elevated

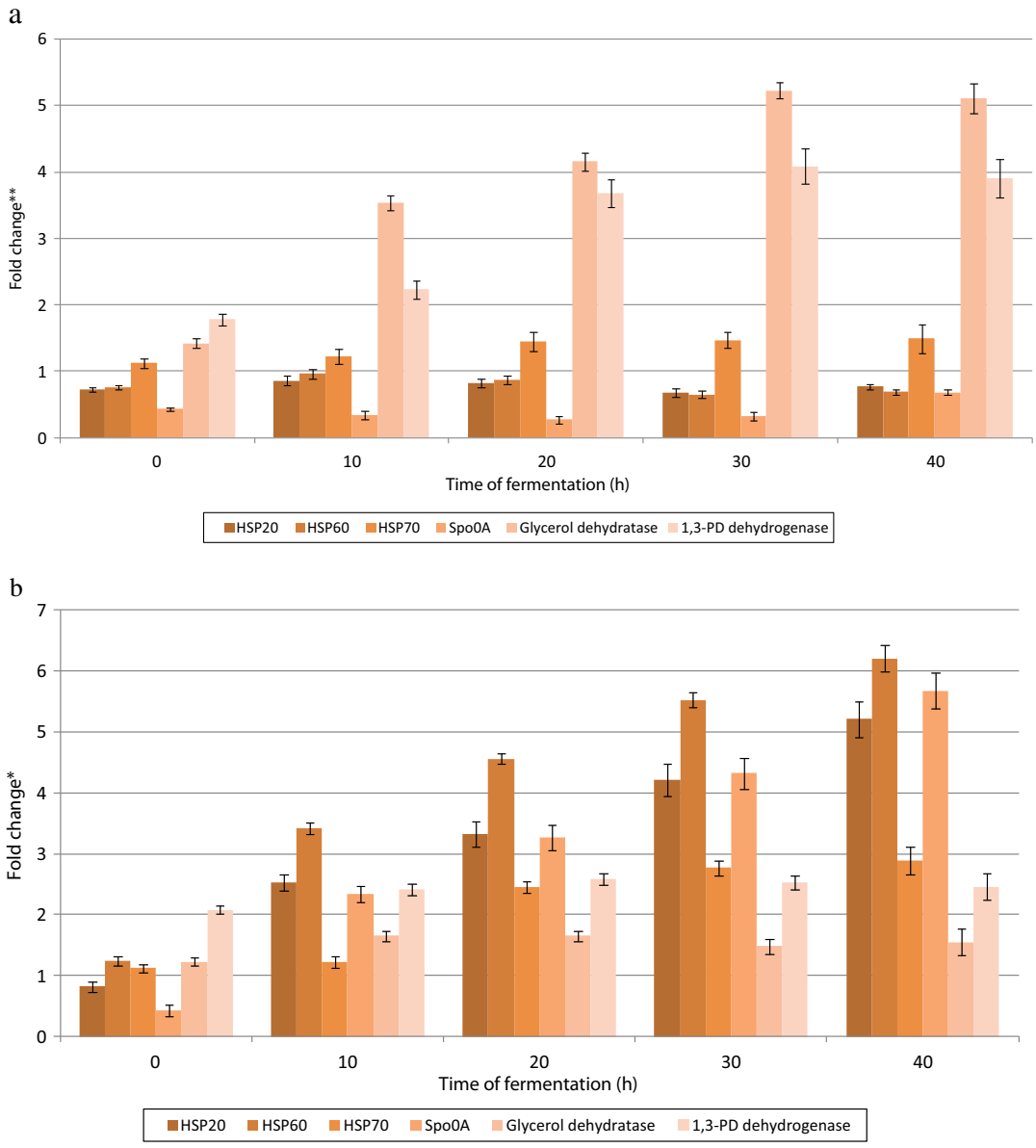

Fig. 2. Proteomic analysis in *C. butyricum* DSP1 (a) neutral conditions and (b) stress conditions. **Fold change in selected proteins in samples from batch fermentation with 90 g/L crude glycerol; *fold change in selected proteins in samples from batch fermentation with 170 g/L crude glycerol.

concentration despite their defensive activity. That progressed until an increase in the Spo0A was observed, as the synthesis of 1,3-PD stopped, leading to sporulation and the arrest of the cells' life-sustaining functions [31]. It should be emphasized that by the time 1,3-PD synthesis came to a halt there was a high concentration of the diol as well as organic acids inside and outside the cells. A simultaneous presence of various stressors, which in this context may be referred to as multifunctional environmental stress, very likely added to the impact of unfavorable factors on the bacterial cells as was further confirmed by the observation of cell conglomerates (Fig. 1b), whose formation is believed to be a protective measure [32].

5. Concluding remarks

This study of the effect of glycerol on the growth and metabolism of *C. butyricum* DSP1 demonstrated that the maximum substrate concentrations that do not inhibit the metabolic activity of bacterial cells are 90 g/L and 70 g/L for a pure and a crude glycerol, respectively. It was also found that an adaptation procedure involving the passage of metabolically active biomass from a fermentation medium with a lower concentration of crude glycerol to media with successively greater concentrations allows breaking the barrier of high osmotic pressure and affords a 1,3-PD concentration of 74 g/L in a batch culture operation. Due to multifunctional environmental stress affecting the fermentation medium there is a need to determine maximum non-inhibiting concentrations for 1,3-PD and other metabolites in the next stage of research in order to define the production capacity of *C. butyricum* DSP1 and to advance an understanding of its resistance to stress factors.

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Conflict of interest

The author declare that has no competing interests.

References

[1] Szymanowska-Powałowska D, Białas W. Scale-up of anaerobic 1,3-propanediol production by *Clostridium butyricum* DSP1 from crude glycerol. BMC Microbiol 2014;14:45. http://dx.doi.org/10.1186/1471-2180-14-45.

[2] Nicolaou SA, Gaida SM, Papoutsakis ET. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 2010;12:307–31. http://dx.doi.org/10.1016/j.ymben.2010.03.004.

[3] Papanikolaou S, Fakas S, Fick M, Chevalot I, Galiotou-Panayotou M, Komaitis M, et al. Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass Bioenergy 2008;32:60–71. http://dx.doi.org/10.1016/j.biombioe.2007.06.007.

[4] Bahl H, Dürre P. Clostridia biotechnology and medical applications. Wiley-Vch Verlag. GmbH; 2001.

[5] Kubiak P, Leja K, Myszka K, Celińska E, Spychała M, Szymanowska-Powałowska D, et al. Physiological predisposition of various *Clostridium* species to synthesize 1,3-propanediol from glycerol. Process Biochem 2012;47:1308–19. http://dx.doi.org/10.1016/j.procbio.2012.05.012.

[6] Amaral PFF, Ferreira TF, Fontes GC, Coelho MAZ. Glycerol valorization: New biotechnological routes. Food Bioprod Process 2009;87:179–86. http://dx.doi.org/10.1016/j.fbp.2009.03.008.

[7] Chatzifragkou A, Papanikolaou S, Dietz D, Doulgeraki AI, Nychas GJE, Zeng AP. Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. Appl Microbiol Biotechnol 2011;91:101–12. http://dx.doi.org/10.1007/s00253-011-3247-x.

[8] Yang F, Hanna MA, Sun R. Value-added uses for crude glycerol-a byproduct of biodiesel production. Biotechnol Biofuels 2012;5:13. http://dx.doi.org/10.1186/1754-6834-5-13.

[9] Sullivan CJ. Propanediols. Ullmann's encyclopedia of industrial chemistry. Weinheim, Germany: VCH; 1993. p. 163–71.

[10] Nakagawa Y, Tamura M, Tomishige K. Catalytic materials for the hydrogenolysis of glycerol to 1,3-propanediol. J Mater Chem A 2014;2:6688–702. http://dx.doi.org/10.1039/c3ta15384c.

[11] Forsberg CW. Production of 1,3-propanediol from glycerol by *Clostridium acetobutylicum* and other *Clostridium* species. Appl Environ Microbiol 1987;53:639–43.

[12] Kaur G, Srivastava AK, Chand S. Advances in biotechnological production of 1,3-propanediol. Biochem Eng J 2012;64:106–18. http://dx.doi.org/10.1016/j.bej.2012.03.002.

[13] Papanikolaou S, Ruiz-Sánchez P, Pariset B, Blanchard F, Fick M. High production of 1,3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. J Biotechnol 2000;77:191–208. http://dx.doi.org/10.1016/S0168-1656(99)00217-5.

[14] Metsoviti M, Paramithiotis S, Drosinos EH, Galiotou-Panayotou M, Nychas GJE, Zeng AP, et al. Screening of bacterial strains capable of converting biodiesel-derived raw glycerol into 1,3-propanediol, 2,3-butanediol and ethanol. Eng Life Sci 2012;12:57–68. http://dx.doi.org/10.1002/elsc.201100058.

[15] Drożdżyńska A, Pawlicka J, Kubiak P, Kośmider A, Pranke D, Olejnik-Schmidt A, et al. Conversion of glycerol to 1,3-propanediol by *Citrobacter freundii* and *Hafnia alvei* — Newly isolated strains from the *Enterobacteriaceae*. New Biotechnol 2014;31:402–10. http://dx.doi.org/10.1016/j.nbt.2014.04.002.

[16] Szymanowska-Powałowska D, Drożdżyńska A, Remszel N. Isolation of new strains of bacteria able to synthesize 1,3-propanediol from glycerol. Adv Microbiol 2013;3:171–80.

[17] Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54. http://dx.doi.org/10.1006/abio.1976.9999.

[18] Petitdemange E, Dűrr C, Andaloussi SA, Raval G. Fermentation of raw glycerol to 1,3-propanediol by new strains of *Clostridium butyricum*. J Ind Microbiol 1995;15:498–502. http://dx.doi.org/10.1007/BF01570021.

[19] Ringel AK, Wilkens E, Hortig D, Willke T, Vorlop KD. An improved screening method for microorganisms able to convert crude glycerol to 1,3-propanediol and to tolerate high product concentrations. Appl Microbiol Biotechnol 2012;93:1049–56. http://dx.doi.org/10.1007/s00253-011-3594-7.

[20] Asad-ur-Rehman A, Saman WRG, Nomura N, Sato S, Matsumura M. Pre-treatment and utilization of raw glycerol from sunflower oil biodiesel for growth and 1,3-propanediol production by *Clostridium butyricum*. J Chem Technol Biotechnol 2008;83:1072–80. http://dx.doi.org/10.1002/jctb.1917.

[21] Chatzifragkou A, Dietz D, Komaitis M, Zeng AP, Papanikolaou S. Effect of biodiesel-derived waste glycerol impurities on biomass and 1,3-propanediol production of *Clostridium butyricum* VPI 1718. Biotechnol Bioeng 2010;107:76–84. http://dx.doi.org/10.1002/bit.22767.

[22] Samul D, Leja K, Grajek W. Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol 2014;64:891–8. http://dx.doi.org/10.1007/s13213-013-0767-x.

[23] Mu Y, Teng H, Zhang DJ, Wang W, Xiu ZL. Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparation. Biotechnol Lett 2006;28:1755–9. http://dx.doi.org/10.1007/s10529-006-9154-z.

[24] Wilkens E, Ringel AK, Hortig D, Willke T, Vorlop KD. High-level production of 1,3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a. Appl Microbiol Biotechnol 2012;93:1057–63. http://dx.doi.org/10.1007/s00253-011-3595-6.

[25] Rywińska A, Rymowicz W, Żlarowska B, Wojtatowicz M. Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation. Food Technol Biotechnol 2009;47:1–6.

[26] Jun SA, Moon C, Kang CH, Kong SW, Sang BI, Um Y. Microbial fed-batch production of 1,3-propanodiol using raw glycerol with suspend and immobilized *Klebsiella pneumoniae*. Appl Biochem Biotechnol 2010;161:491–501. http://dx.doi.org/10.1007/s12010-009-8839-x.

[27] Hirschmann S, Baganz K, Koschik I, Vorlop KD. Development of an integrated bioconversion process for the production of 1,3-propanediol from raw glycerol waters. Landbauforsch Voelkenrode 2005;55:261–7.

[28] González-Pajuelo M, Andrade JC, Vasconcelos I. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. J Ind Microbiol Biotechnol 2004;31:442–6. http://dx.doi.org/10.1007/s10295-004-0168-z.

[29] Sullivan L, Benett GN. Proteome analysis and comparison of *Clostridium acetobutylicum* ATTC 824 and Spo0A strain variants. J Ind Biotechnol 2006;33:298–308. http://dx.doi.org/10.1007/s10295-005-0050-7.

[30] Shaoming M, Yuanming L, Tianrui Z, Jinshan L, Guanhui B, Yan Z, et al. Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. J Proteome Res 2010;9:3046–61. http://dx.doi.org/10.1021/pr9012078.

[31] Dürre P, Hollergschwandner C. Initiation of endospore formation in *Clostridium acetobutylicum*. Anaerobe 2004;10:69–74. http://dx.doi.org/10.1016/j.anaerobe.2003.11.001.

[32] Camesano TA, Natan MJ, Logan BE. Observation of changes in bacterial cell morphology using tapping mode atomic force microscopy. Langmuir 2000;16:4563–72. http://dx.doi.org/10.1021/la990805o.