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Intensified crude glycerol conversion to butanol by immobilized *Clostridium pasteurianum*

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Abstract

Butanol production from glycerol was investigated through *Clostridium pasteurianum* entrapped into polyvinyl alcohol particles. Using an optimized system, batch and repeated batch fermentations with free and entrapped cells were performed, respectively. In both systems, glycerol samples of different purity were tested. In repeated batch fermentations, process time decreased from 19.5 to 2.7 hours and butanol productivity increased 6.3 times (3.08 $g.L^{-1}.h^{-1}$) compared with the free-cell process, using pure glycerol. In the case of glycerol from biodiesel production, butanol productivity of 2.90 and 1.76 $g.L^{-1}.h^{-1}$ was achieved from glycerol 01 and glycerol 02, respectively. No cell growth or solvent production was observed when the same glycerols were used in free-cell fermentations.

Keywords

butanol, crude glycerol, *Clostridium pasteurianum*, immobilization

1. Introduction

n-Butanol, ($C_4H_{10}O$), has a wide utility in the chemicals industry. Butanol has physical properties that make it easy to transport and store: comparatively low vapour pressure, large liquid range and low corrosivity. The intermediate oxidation level of alcohols makes them useful starting materials for building a range of chemical functionality. Thus butanol is a useful solvent and a very convenient source of C4, and is frequently applied to ester and ether forming reactions. Butanol has found application in commodity chemicals, materials and textiles e.g. in the production of paints (as a solvent and 2-butoxyethanol), butyl acrylate (bulk chemical) and plasticizer dibutyl sebacate. In addition, it has a great potential to become a new biofuel due to its advantages over other simple organic bioproducts [1]. Compared with ethanol, it has 30% higher content of energy and can be used in gasoline cars without special modifications of the engine [2, 3]. Biobutanol can be produced by several strains of the *Clostridium* genus, for example *C. acetobutylicum*, *C. beijerinckii*, or *C. pasteurianum* [4]. From an ecological perspective it is essential to use waste substrates for biofuel formation such as molasses, bran hydrolysate or glycerol.

Glycerol is a natural polyol with wide application in the pharmaceutical, cosmetic and food industries [5]. Today, most glycerol comes from biodiesel production, where it is formed as the main by-product. During transesterification, 10% (v/v) of fats is converted to crude glycerol. Increased world biodiesel production has also influenced glycerol stock and prices, which has allowed its application as a carbon source [6]. There are various species, including *Citrobacter, Klebsiella, Clostridium, Lactobacillus*, which utilize glycerol [7]. However, butanol production from glycerol is typical only for *Clostridium pasteurianum*.

48 *C. pasteurianum* is a strictly anaerobic bacteria which digests glycerol and generates
49 butanol as a main product with 1,3-propanediol, ethanol, butyrate, acetate, lactate, CO_2 and
50 H_2 as by-products [8, 9]. Butanol synthesis is the energetically preferred pathway, but 1,3-
51 propanediol production is essential for NAD^+ regeneration [10].

52 The main problem with the application of crude glycerol to industrial fermentations
53 is the inhibitory effect caused by impurities. After transesterification, glycerol usually
54 contains different concentrations of methanol (residues of the biodiesel methylation
55 process), sulphate or chloride salts, residues of free fatty acids (FFA), fatty acid methyl
56 esters (FAME) and soaps (as a by-product of the hydroxide transesterification catalyst and
57 FFA) [5, 11]. The first option for overcoming this inhibitory effect is crude glycerol
58 purification, which negatively influences the price of substrate [6]. A more interesting
59 choice is the mutation or adaptation of wild-type strains to crude glycerol, this approach has
60 been reported for *Clostridium pasteurianum* [12, 13]. On the other hand, the strain is
61 adapted only to one specific type of crude glycerol, and other substrate batches may also be
62 toxic for adapted strains. Therefore other, more general options, such as the immobilization
63 of microorganisms and their protection by gel matrices, might overcome the toxic effect of
64 the crude glycerol.

65 Immobilization is based on the fixation of biocatalysts into or onto various
66 materials, for example natural wooden scobs, gelatine, agarose or synthetic polyurethane,
67 polyacrylamide or polyvinyl alcohol (PVA) [14]. The resulting improvements of the
68 bioprocess can include biocatalyst recycling in a repeated batch or continuous processes, or
69 the protection of the biocatalyst against environmental effects [15-17]. Moreover,
70 immobilization often increases yields and productivities as it facilitates a high
71 concentration of the biocatalyst, as well as improving the process and storage stability. In

addition, immobilized biocatalysts often lower sensitivity to contamination, allowing in some cases non-sterile process conditions [18]. Improvement of crude glycerol utilization by immobilized biocatalysts has already been reported for hydrogen [19], 1,3-propanediol [20], and butanol production [9, 21].

In this work, entrapment into PVA particles was tested [14]. PVA is a very suitable entrapment material because of its high tolerance towards a wide range of temperatures (10–50 °C) [22] and pHs (2.3-9.0) [23, 24]. In addition, PVA is not biodegradable and not toxic for cells or enzymes. The used entrapment method was characterized the mild conditions for the preparation of particles and the protective potential for the biocatalyst. The resulting PVA particles have a high inner porosity for cell colonization, excellent physical and mechanical properties [25], a unique shape allowing sufficient diffusion of the substrate and products in and out of the particles, but also easy separation from the liquid media by sieve [26]. Several publications have already described the positive impact of these particles on fermentations in unfavourable conditions [14].

This work was focused on the entrapment of *C. pasteurianum* DSM 525 into PVA particles. Glycerol of different purities was tested for butanol production by free and entrapped cells.

2. Materials and Methods

2.1. Strain

C. pasteurianum DSM 525 was obtained from the German Collection of Microorganisms. The strain was stored on Petri dishes with reinforced *Clostridia* agar media (RCM), (MERCK, Germany) in an anaerobic chamber (BACTRON I, Shel Lab, USA) with an inert atmosphere (90% N_2, 5% CO_2, 5% H_2) at 34 ºC. The strains were periodically re-plated on a weekly basis.

2.2. Media

Inoculation media (per litre of distilled water): 30 g pure glycerol (Centralchem, Slovakia); 2.5 g KH_2PO_4; 2.5 g K_2HPO_4, 0.02 g $CaCl_2$; 1 g yeast extract; 5 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 5 mg $FeSO_4.7H_2O$; 2 mL SL 7. Media were placed in reagent bottles (Pyrex®, SciLabware Limited, UK), closed with rubber lids (Suba-Seal®, Sigma–Aldrich Co., USA). The media were sparged with nitrogen (N_2) for 15 min and then autoclaved (120 °C, 20 min). $MgSO_4.7H_2O$ was autoclaved and added separately. $FeSO_4.7H_2O$ was filtered through a sterile filter (Filtropur, 0.2 μm cellulose acetate membrane, Sarstedt, Germany) into the medium after sterilization.

Production media were composed of (per litre of distilled water): 0.5 g KH_2PO_4; 0.5 g K_2HPO_4, 0.02 g $CaCl_2$; 1 g yeast extract; 5 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 5 mg $FeSO_4.7H_2O$; 2 mL SL 7; 200 μL silicone antifoam (DowCorning® 1510, VWR, UK); 40–60 g glycerol of different purity (Table 1). The media were autoclaved (120 °C, 20 min) in fermenters and then sparged with 0.1 vvm of N_2 for 1 hour. $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ were added, as mentioned above.

SL7 solution was composed of (per litre of distilled water): 1 mL HCl (25%) ; 0.07 g $ZnCl_2$; 0.1 g $MnCl_2.4H_2O$; 0.6 g H_3BO_3; 0.2 g $CoCl_2.6\ H_2O$; 0.2 g $CuCl_2.2H_2O$; 0.2 g $NiCl_2.6H_2O$; 0.04 g $Na_2MoO_4.2\ H_2O$. Prepared solution was stored at 4 °C.

Samples of glycerol from biodiesel production were kindly provided by Meroco a.s., company (Leopoldov, Slovakia).

Insert table 1

2.3. Inoculum preparation

Pre-inoculum was prepared in 10 ml of inoculation medium with a single colony of *C. pasteurianum* DSM 525. The preculture was incubated for 12–15 hours at 34 °C and 200

120 rpm in the anaerobic chamber. The biomass (5% v/v) was then placed in 50 ml of inoculum
121 medium in 100 ml Durham bottles (Fisherbrand®, Fisher Scientific, USA) and cultivated in
122 the above-mentioned conditions. After reaching optical density of 3.0 (approx. 9 hours) the
123 culture was used as inoculum for fermenters.

124 **2.4. Fermentations with free cells**

125 The batch fermentations with free cells were performed in 1.3 L laboratory
126 fermenter (BioFlo® 115, New Brunswick, USA) with 1 L of production medium at 34 °C,
127 200 rpm and pH 6 (maintained by 2 M KOH). Each experiment was sparged with 0.1 vvm
128 N_2 through filter (Millex®-FG, 0.2 μm PTFE, Millipore) and then inoculated (5% v/v).

129 **2.5. Strain immobilization**

130 Bacteria for immobilization were prepared as described above. A total of 50 ml of
131 cultivated cells were centrifuged at 4 °C and 2935 g for 15 minutes. The pellet was mixed
132 with 5 ml of sterile anaerobic distilled water. 10 g of PVA and 8 g of polyethylene glycol
133 (PEG) were added to 77 ml of distilled water and mixed in water bath at 90°C for 30
134 minutes. Then PVA gel was cooled to 40°C and mixed with 5 ml of the cells. Using the
135 Lentiprinter® device, PVA particles were prepared and dried by the air at 40°C for 20
136 minutes. The last step was the particles stabilization to 0,1 M Na_2SO_4 for 30 minutes.
137 Process was repeated 3 times to get 100 g of PVA particles. Immediately after biomass
138 immobilization were particles used for repeated batch fermentations.

139 **2.6. Fermentations with immobilized cells**

140 Repeated batch fermentations with immobilized particles were made with the same
141 medium as free cell fermentations, with the addition that 100 g of particles with
142 immobilized cells were placed into the fermenter with the production medium in a laminar
143 box. After that, the fermenter (working volume 1 L, agitation 250 rpm) was inoculated with

144 free-cell culture (5% v/v) at the beginning of the first repeated batch. After optimization,
145 described in section 3.2, the second repeated batch was also inoculated. After reaching a
146 stationary phase of growth, the whole medium with free cells was separated by sieve and
147 replaced with a fresh one, while PVA particles with entrapped cells remained in the
148 fermenter. A total of 102 consecutive repeated batches were made, during which different
149 purity aliquots of glycerol were tested.

150 **2.7. Analytical assays**

151 The concentration of the free cells and released from PVA particles during
152 fermentations (OD) was measured with a spectrophotometer (BioSpectrometer®,
153 Eppendorf, Germany) at 600 nm. Glycerol and metabolism products were analysed by
154 HPLC (Agilent Technologies 1260 Infinity, USA) on an 8 μm, 250 x 8 mm column with a
155 Polymer IEX H^+ form (WATREX, Czech Republic) protected by a guard column (polymer
156 IEX H^+ form 8 μm, 40 × 8 mm). The temperature in the column was 50 °C. As the mobile
157 phase, 9 mM H_2SO_4 with flow rate 1 $mL.min^{-1}$ was used. The substrate/products were
158 identified by RI detector at 40 °C and UV detector at 258 nm. Values in graphs are below
159 3% of standard deviation. Butanol productivity was calculated as the final butanol
160 concentration divided by the fermentation duration. Butanol yield was calculated as grams
161 of product per gram of utilized glycerol.

162 **2.8. Electron microscopy**

163 Microphotographs of the PVA particles with entrapped cells were performed with
164 an electron microscope JEOL 7500F (JEOL, Japan) using the CRYO system Quorum
165 (Quorum Technologies, UK). Before measuring, samples were frozen by liquid nitrogen,
166 cut in vacuum, sublimed at -90 °C for 10 minutes and metallized using a
167 palladium/platinum mixture. Cryo-mode measurement was made in vacuum at -140 °C.

3. Results and Discussion

The first part of the work was focused on optimizing conditions for *C. pasteurianum* DSM 525 with free cells. Then the inhibition effect of crude glycerols was investigated. Obtained results were later applied in the process with an immobilized microorganism.

3.1. Batch fermentation with free cells

The original medium for free-cell batch fermentations was adopted from previous research [10]. After a series of experiments, (data not shown) the best conditions were chosen as 34 °C, 200 rpm and pH 6. The most suitable substrate concentration was 60 $g.L^{-1}$ of pure glycerol (where no unutilized substrate was left in the medium), where 12.3 $g.L^{-1}$ of butanol was obtained after 20 hours of fermentation (Fig. 1). Butanol yield and productivity represented 0.2 $g.g^{-1}$ and 0.56 $g.L^{-1}.h^{-1}$, respectively. Higher concentrations of substrate were not utilized, probably due to the toxic effect of butanol on *C. pasteurianum*. Approx. 12–13 $g.L^{-1}$ seemed to be the maximum limit that the used strain could tolerate (12.9 $g.L^{-1}$ of butanol was obtained by utilizing 90 $g.L^{-1}$ of substrate). The butanol inhibition effect is a known phenomenon, which is widely studied and described [12, 27]. A similar butanol toxic level (12.6 $g.L^{-1}$) was found with a mutant strain of *C. pasteurianum* [13].

Insert Figure 1

In the case of crude glycerol samples (initial concentration 40 $g.L^{-1}$) no cell growth was observed in 24 hours of fermentation under the same conditions. As mentioned above, crude glycerol utilization by wild types of microorganisms is problematic, therefore different approaches such as mutant or adapted strains are applied [12, 13]. However, the adaptation of *C. pasteurianum* to crude glycerol in our laboratories was not successful. Therefore, immobilization of cells by entrapment in PVA gel, exploiting the protective effect of the gel matrix, was tested in further experiments.

3.2. Optimization of repeated batch fermentations with entrapped *C. pasteurianum*

As mentioned above, PVA gel immobilization can be applied to different enzymes or microorganisms. However, this entrapment technique is an aerobic process [28]. Therefore it is problematic to immobilize strictly anaerobic strains using this technique. The modified protocol for *C. acetobutylicum* [25] was applied to *C. pasteurianum*. Modifications were mainly focussed on *C. pasteurianum* contamination prevention and culture stabilization. Detailed description of tested modifications can be found in supplementary material.

In summary, the first two repeated batches were inoculated with free cells, which created selective conditions for entrapped *C. pasteurianum* growth. In the next series of repetitions, spores from the inner part of particles and cells adsorbed to their surface (from free cell inoculum), started to gradually colonize PVA particles and no contamination was detected (Fig. A.3, for details see supplementary material).

To experimentally confirm the contribution of entrapped biomass to the fermentation process, the repeated batches with empty PVA particles (without any entrapped biomass) and addition of free cells as inoculum was tested. Also in this case, gradual increase of productivity was observed. After 30^{th} repetition process started to be stable (Fig. A.5). In contrast, experiment with entrapped cells continued to improve up to 56^{th} repetition and then achieved 1,5 times higher volumetric productivity. Since the difference between experiments was only in the addition of spores in entrapment, positive impact was caused by spore germination inside the particles and therefore higher biomass content in the process. Similar conclusion was reached in work with *C. tyrobutyricum* [29].

3.3. Repeated batch fermentations with pure glycerol

As mentioned in section 3.1, media used in free-cell fermentations, contained the initial glycerol concentration 60 $g.L^{-1}$. However, repeated exposure of immobilized cells to limiting butanol concentrations led to decreases in cell growth and butanol productivity (Fig. A.6). Therefore 50 $g.L^{-1}$ was chosen for the next application. In total 68 repeated batches were made by the entrapped *C. pasteurianum* with pure glycerol as a carbon source (Table 2). A significant decrease in fermentation time was observed between the first and the second repetition due to the added inoculum, as mentioned above (Fig. 2). Up to the fifth batch, fermentation time and butanol productivity were relatively stable, due to the dominating acidification phase of metabolism. After that, a significant reduction of the fermentation time began. Compared with the first repetition, fermentation time in the tenth repetition decreased from 19.4 to 6.1 hours and butanol productivity increased from 0.44 to 1.08 $g.L^{-1}.h^{-1}$. After that, the process duration gradually decreased until the 56th batch and was quite stable for the rest of the repetitions.

Insert Figure 2

Average fermentation time after the 58th batch reached 2.69 ± 0.05 hours and butanol productivity increased up to 3.08 ± 0.33 $g.L^{-1}.h^{-1}$ which is 6.3 times higher compared with the free-cell fermentation (Table 2). Butanol concentration stabilized at 10.40 ± 0.81 $g.L^{-1}$, giving an average yield of 0.21 ± 0.03 $g.g^{-1}$, which is almost identical to the free cells. The best results were observed in the 57th repetition, where butanol productivity, concentration and yield reached values of 3.22 $g.L^{-1}.h^{-1}$; 11.52 $g.L^{-1}$ and 0.21 $g.g^{-1}$, respectively (Fig. 3). Compared with the first batch, where the lag phase took almost 10 hours, the 57th batch fermentation had almost no lag phase. This was a consequence of *C. pasteurianum* gradual colonization in PVA, which led to high biomass density inside the

238 particles. Moreover, the particles served also as inoculum, when new cells leaked out from
239 the surface to the medium and served as inoculum for next batch. The obtained optical
240 densities in repeated batches were almost identical as with free cells but in much shorter
241 time, which confirmed cell leakage out of the particles (Table 2). It is important to add that
242 measured OD represented amount of leaked cells but didn't correspond to the overall
243 biomass present in the fermenter. The majority of the cells were entrapped in the particles.
244 To confirm the above-mentioned colonization, PVA particles were subjected to electron
245 microscopy, after propagation of the biomass. Fig. 4 displays the inner structure of PVA
246 lens at the beginning of the experiment (0.4 $g.L_{gel}^{-1}$ of immobilized biomass) and later,
247 when maximal butanol productivity was reached.

248 Furthermore, there were no observed changes in PVA particles mechanical stability
249 after 100 repetitions. This is in accordance with previously reported experiments, where
250 *Zymomonas mobilis*, massive ethanol and CO_2 producer, was immobilized and after 30
251 repetitive batches the productivity and mechanical stability of particles remained
252 unchanged [30].

253 *Insert Figure 3*

254 As reported before [25], residual amounts of organic acids help to shift the
255 metabolism from acidogenic to solventogenic phases. No inhibition was observed during
256 the medium change in the fermenter, probably caused by the positive effect of the PVA
257 hydrogel biomass protection.

258 *Insert Table 2*

259 Compared to previous findings, the obtained average butanol productivity was 6.4 times
260 higher than continuous fermentation with immobilized cells of *C. pasteurianum* NRRL B-
261 598 (0.48 ± 0.04 $g.L^{-1}.h^{-1}$) on corncob residues [31] and 2.5 times higher than the repeated

262 fed-batch fermentations (1.21 $g.L^{-1}.h^{-1}$) with *C. acetobutylicum* DSM 1731, where
263 entrapment of anaerobic bacteria into the PVA was used [25]. Although Gallazzi *et al.* [32]
264 achieved butanol productivity of 4.2 $g.L^{-1}.h^{-1}$ (36% higher than in this work), the average
265 butanol concentration was 9.5 $g.L^{-1}$ (0.9 $g.L^{-1}$ less, compared with the presented data).
266 Moreover, the experiment was conducted in a packed-bed fermenter with fully packed corn
267 stover pieces in a continuous mode, which represents higher investment costs for
268 equipment than the application of PVA particles to stirred reactors with quite low
269 immobilization (10% w/v) load.

270 *Insert Figure 4*

271 **3.4. Repeated batch fermentations with crude glycerol**

272 Although the results with pure glycerol were very promising, more attractive – as
273 pointed out above – is the application of crude glycerol in the process, to reduce the input
274 cost of the substrate. During the experiment, two types of crude glycerol were applied
275 (Table 1) after the repeated batches with pure glycerol. Therefore the first fermentation
276 with glycerol 01 was repetition number 69. For both glycerol types two different initial
277 concentrations were tested (Table 3). Compared with free-cell fermentations, entrapped
278 cells could utilize both crude glycerol samples and produce butanol. In the case of glycerol
279 01, final butanol concentrations and yields were comparable with those of pure glycerol.
280 On the other hand, butanol productivity in all cases decreased, probably due to inhibition by
281 impurities. Final values (for 50 $g.L^{-1}$ of initial glycerol) were 2.42 ± 0.23 and 1.73 ± 0,36
282 $g.L^{-1}.h^{-1}$ for glycerol 01 and glycerol 02, respectively, which represented a decrease of 0.66
283 and 1.35 $g.L^{-1}.h^{-1}$ compared to experiments with pure glycerol. According to [33], methanol
284 and salts have no negative influence on cell growth or butanol production. On the other
285 hand, fatty acids with a higher degree of unsaturation are major contributors having a

strong inhibitory effect. Results in this work support this theory, because glycerol 02 also contained residues of FFA and FAME (Table 1). Moreover, sodium or potassium salts of fatty acids (soaps) are usually part of the ash in crude glycerol, which could explain the productivity decrease in both experiments. Nevertheless, the PVA gel protective effect enabled *C. pasteurianum* utilization of glycerol to butanol even in conditions that are toxic for the free cells. This corresponds with other publications, where PVA particles were used for wastewater treatment of industrial waters containing high concentrations of salts [14, 16] or radioactive elements [17]. Even if inhibitors caused a gradual decrease in activity, it is possible to implement a revitalization step for biomass restoration [17, 34]. For comparison, *C. pasteurianum* ATCC 6013 immobilized on Amberlite was able to form 8.84 $g.L^{-1}$ of butanol after 120 h (productivity 0.074 $g.L^{-1}.h^{-1}$). Moreover, the maximum tolerable concentration of crude glycerol for butanol formation was 25 $g.L^{-1}$ [9]. In both cases reported in this publication, entrapped cells were active at 40 and 50 $g.L^{-1}$. In the case of the mutant strain of *C. pasteurianum* MNO6, butanol productivity and yield were 1.8 $g.L^{-1}.h^{-1}$ and 0.20 $g.g^{-1}$ [13], which correspond to results with glycerol 02. However, in the process gas-stripping was used [13], which decreased the butanol's inhibitory effect and therefore enhanced production.

Insert Table 3

4. Conclusion

This study was focused on butanol production from biodiesel-derived glycerol using entrapped *C. pasteurianum*. Immobilized cells were able to colonize PVA particles, which led to a significant decrease in fermentation time and an improvement in butanol productivity. With pure glycerol and the crude glycerol 01 and glycerol 02, butanol productivities of 3.08 ± 0.33; 2.42 ± 0.23; and 1.73 ± 0.36 $g.L^{-1}.h^{-1}$ were achieved,

310 respectively. Butanol yield was stable in all experiments. The results obtained confirmed
311 the positive impact of the PVA gel immobilization method on microorganism resistance
312 against crude glycerol impurities and an improvement of process parameters.
313
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Table captions:

Table 1 Crude glycerol composition used in experiments

Table 2 Comparison of process parameters in batch and repeated batch fermentations

Table 3 Comparison of repeated batch process parameters using different purity glycerol batches

413 **Figure captions:**

414 **Fig.1** Batch fermentation with free *C. pasteurianum* on pure glycerol.

415 **Fig. 2** Fermentation time and volumetric butanol productivity (Q_p) for repeated batches
416 with pure glycerol using immobilized *C. pasteurianum*.

417 **Fig. 3** Comparison of the 1st (left) and the 57th (right) repetition of immobilized *C.*
418 *pasteurianum*.

419 **Fig. 4** SEM images (2500 x) of PVA particles inner structure. Empty porous structure (left)
420 of particles and after colonization by *C. pasteurianum* (right).

421

422 **Table 1**

423

	Glycerol 01	Glycerol 02
FFA (%)	0.000	0.002
FAME (%)	0.000	0.009
Methanol (%)	0.25	20.70
Density ($kg.m^{-3}$)	1277.00	1098.00
Water content (%)	9.98	25.80
Ash (%)	5.20	2.60
Glycerol content (%)	89.55	52.66

424 **Table 2**

	Free-cell batches		Repeated batches (immobilized cells)			
			1st batch	10th batch	57th batch	56th-68th batches
Residual glycerol ($g.L^{-1}$)	5.21	3.31	2.69	14.92	9.25	10.46±1.74
Produced butanol ($g.L^{-1}$)	9.49	12.28	8.62	7.72	8.59	8.38±0.86
1,3 propanediol ($g.L^{-1}$)	1.33	4.05	0.93	1.57	3.21	3.22±0.30
Initial glycerol ($g.L^{-1}$)	50.57	60.57	50.47	50.57	50.50	50.51±2.77
OD_{600nm}	17.27	20.12	20.49	10.40	16.29	15.11±0.85
Fermentation time (h)	19.50	22.00	19.42	7.13	2.67	2.69±0.05
Butanol productivity ($g.L^{-1}.h^{-1}$)	0.49	0.56	0.44	1.08	3.22	3.12±0.33
Butanol yield ($g.g^{-1}$)[a]	0.21	0.21	0.18	0.22	0.21	0.21±0.02

[a] Yield of butanol calculated as final butanol concentration divided by concentration of utilized glycerol

425

426 **Table 3**

	Repeated batches (immobilized cells)				
	Pure Glyc. 56^{th}-68^{th} batches	Glyc.01 69^{th}-77^{th} batches	Glyc.01 78^{th}-83^{rd} batches	Glyc.02 84^{th}-92^{nd} batches	Glyc.02 93^{rd}-101^{st} batches
Residual glycerol ($g.L^{-1}$)	10.46±1.74	6.22±0.43	9.24±0.63	11.52±0.35	17.25±1.55
Produced butanol ($g.L^{-1}$)	8.38±0.86	7.23±0.75	8.41±0.57	5.76±0.59	6.90±0.41
1,3 propanediol ($g.L^{-1}$)	3.22±0.30	3.30±0.15	3.67±0.28	2.56±0.22	3.40±0.88
Initial glycerol ($g.L^{-1}$)	50.51±2.77	40.11±0.47	49.02±1.74	40.68±1.36	51.26±0.59
OD_{600nm}	15.11±0.85	11.99±0.85	9.67±0.60	7.40±0.62	6.55±1.75
Fermentation time (h)	2.69±0.05	2.50±0.08	3.49±0.19	3.29±0.21	4.13±0.71
Butanol productivity ($g.L^{-1}.h^{-1}$)	3.12±0.33	2.90±0.37	2.42±0.23	1.76±0.20	1.73±0.36
Butanol yield ($g.g^{-1}$)[a]	0.21±0.02	0.21±0.03	0.21±0.01	0.20±0.02	0.20±0.01

[a] Yield of butanol calculated as final butanol concentration divided by concentration of utilized glycerol

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