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Batch- and continuous propionic acid production from glycerol using free and immobilized cells of *Propionibacterium acidipropionici*

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Abstract

Propionic acid production from glycerol was studied using *Propionibacterium acidipropionici* DSM 4900 cells immobilized on polyethylenimine-treated Poraver (PEI-Poraver) and Luffa (PEI-Luffa), respectively. Using PEI-Luffa, the average productivity, yield and concentration of propionic acid from 40 $g.L^{-1}$ glycerol were 0.29 $g.L^{-1}.h^{-1}$, 0.74 $mol.mol^{-1}$ and 20.09 $g.L^{-1}$, respectively, after four consecutive recycle-batches. PEI-Poraver supported attachment of 31 times higher amount of cells than PEI-Luffa and produced 20, 28 and 35 $g.L^{-1}$ propionic acid from 40, 65 and 85 $g.L^{-1}$ glycerol, respectively (0.61 $mol_{PA}.mol_{Gly}^{-1}$). The corresponding production rates were 0.86, 0.43 and 0.35 $g.L^{-1}.h^{-1}$, which are the highest reported from glycerol via batch or fed-batch fermentations for equivalent propionic acid concentrations. Using a continuous mode of operation at a dilution rate of 0.1 h^{-1}, cell washout was observed in the bioreactor with free cells; however, propionic acid productivity, yield and concentration were 1.4 $g.L^{-1}.h^{-1}$, 0.86 $mol_{PA}.mol_{Gly}^{-1}$, and 14.5 $g.L^{-1}$, respectively, using immobilized cells in the PEI-Poraver bioreactor. The choice of the immobilization matrix can thus significantly influence the fermentation efficiency and -profile. The bioreactor using cells immobilized on PEI-Poraver allowed the fermentation of higher glycerol concentrations and provided stable and higher fermentation rates than that using free cells or the cells immobilized on PEI-Luffa.

Keywords

Fermentation; Platform chemical; Organic acid; Cell adsorption; Polyethylenimine

1. Introduction

There is a growing interest in sustainable production of chemicals from renewable resources. Propionic acid (PA), a C-3 platform chemical and its calcium-, sodium- and ammonium-salts are widely used as preservatives in feed, food and pharmaceuticals. It is also incorporated into cellulose plastics, herbicides, perfume bases and a range of other products (Rogers et al., 2006). According to the US Department of Energy, propionic acid is among the top 30 candidate platform chemicals employed as building blocks for products with various applications (Werpy et al., 2004). Industrially, propionic acid is produced from petrochemical raw materials via oxo-synthesis utilizing ethylene and carbon monoxide followed by liquid-phase oxidation of the resulting aldehyde, oxidation of propane gas or from propionitrile (Rogers et al., 2006). An alternative renewable route for its production has been extensively investigated since the discovery of propionic acid fermentation (Strecker, 1854); however, this route of production has not gone beyond research scale.

Propionibacteria are Gram positive, facultative anaerobes and can metabolize different carbon sources such as lactose (Jin and Yang, 1998), sucrose (Quesada-Chanto et al., 1994), lactic acid, glucose (Barbirato et al., 1997; Himmi et al., 2000), xylose (Carrondo et al., 1988) and glycerol (Gly) (Barbirato et al., 1997; Bories et al., 2004; Boyaval et al., 1994; Coral et al., 2008; Himmi et al., 2000; Zhang and Yang, 2009b) into propionic acid. With majority of the carbon sources, except glycerol, acetic acid (AA) was obtained as major by-product at a high molar ratio with respect to propionic acid, approaching 0.42 $mol_{AA}.mol_{PA}^{-1}$. Glycerol, in contrast, induces homopropionic acid fermentation, yielding up to 0.9 $mol_{PA}.mol_{Gly}^{-1}$, and acetic acid production was minimized to almost 1 mole for each 30 moles of propionic acid produced or even less (Barbirato et al., 1997; Bories et al., 2004; Coral et al., 2008; Himmi et al., 2000). Glycerol is also a cheap commercially available substrate. It is normally produced as a by-product of fat hydrolysis, ethanol fermentation and more recently from biodiesel

production (Agarwal, 1990; Thompson and He, 2006; Wang et al., 2001). From the perspective of raw materials source and cost, product yield, waste reduction, and easy downstream processing, glycerol is considered an advantageous carbon source.

However, despite many reported and patented processes, strain and media optimizations, no industrial process based on fermentation has been established due to low volumetric productivity, yield and final propionic acid concentration obtained with this route. The low market price of propionic acid also results in a narrow difference with the cost of substrates and necessitates the development of a highly efficient process (Chang, 2011). Up to now, the maximum reported volumetric productivity of propionic acid from glycerol by batch or fed-batch modes of operation was 0.8 $g.L^{-1}.h^{-1}$ with a concentration of 12 $g.L^{-1}$ (Bories et al., 2004), and the maximum concentration was 106 $g.L^{-1}$ obtained at a rate of 0.04 $g.L^{-1}.h^{-1}$ (Zhang and Yang, 2009b), results which indicate the requirement for further optimization.

For stable propionic acid production, fermentations with immobilized cells are favored over those with free cells. Immobilization offers many advantages including enhanced volumetric productivity caused by a high cell density of adapted cells (Feng et al., 2011; Huang et al., 2002), reduced need for nitrogen sources which lowers the fermentation cost, and generation of mutant strains with higher tolerance to the inhibitory effect of propionic acid and less by-products formation (Suwannakham and Yang, 2005; Zhang and Yang, 2009a). Furthermore, under continuous mode of operation, bioreactors harboring immobilized cells can operate at high dilution rates without cell washout (Paik and Glatz, 1994). Different immobilization techniques have been reported; however, adsorption on a solid support and entrapment inside a polymer matrix are the most studied. Although providing improved volumetric productivities, entrapment is characterized by poor mechanical stability and is less suitable for growth-associated products such as propionic acid. Adsorption, in contrast, allows continuous release of dead cells and replacement with active ones (Bruno-Barcena et al., 2000; Lewis and Yang, 1992b). However, for efficient cell immobilization via adsorption, different factors should be

considered including matrix structure and characteristics, the microorganism, and the immobilization environment (Goller and Romeo, 2008; Oliveira et al., 2000). Surface modification to provide electrostatic forces (cationic polymers) could also be applied to enhance immobilization efficiency (D´Souza et al., 1986; Guoqiang et al., 1992; Senthuran et al., 1997).

In the present study, production of propionic acid from glycerol was investigated using cells of *P. acidipropionici* DSM 4900 immobilized on two matrices, Luffa and Poraver treated with a cationic polymer, polyethylenimine (Guoqiang et al., 1992; Senthuran et al., 1997). Luffa is the fibrous mature dried fruit of *Luffa cylindrica* available cheaply in most tropical countries, while Poraver is a trade name for foamed highly porous recycled glass beads. Immobilized-cell bioreactors were operated with different glycerol concentrations in recycle-batch and continuous modes of operation, respectively, to determine process efficiency and stability.

2. Materials and Methods

2.1. Materials and media composition

Poraver beads (6-8 mm diameter) were obtained from Dennert Poraver (Postbauer-Heng, Germany) while Luffa was purchased from a local supplier. Polyethylenimine (PEI, average MW ~25,000 by light scattering, average Mn ~10 000 by GPC, branched [$H(NHCH_2)_nNH_2$]), ammonium hydroxide (28%), L-cysteine HCl, anhydrous (98%) and glycerol (99%) were procured from Sigma-Aldrich (St Louis, MO, USA). Bacto yeast extract was a product of Difco (BD laboratories, Detroit, Michigan, USA).

2.2. Microorganism and culture conditions

Propionibacterium acidipropionici DSM 4900 was grown anaerobically in medium (at pH 7) containing per liter: 10 g yeast extract, 40 g glycerol, 2.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 0.25

g L-cysteine HCl. For preparation of the pre-culture, 20 mL of this medium was boiled in 30-mL serum bottles, bubbled with oxygen free nitrogen and autoclaved at 121 ˚C for 20 min. One milliliter of stock culture in glycerol was added to the sterile medium and incubated at 30 ˚C for 5 days. The resulting culture was used to inoculate another 20 mL of medium (5% $v.v^{-1}$) but incubated for 3 days only to reach stationary phase (OD_{620nm} of 7.3) before being used as inoculum for propionic acid production and cell immobilization experiments.

For propionic acid production, the same medium was used with varying glycerol concentrations. In case of the fermentations with immobilized cells, phosphate buffer was omitted from the fermentation medium to avoid interaction with PEI.

2.3. Free cell batch fermentation using P. acidipropionici

Freshly prepared inoculum was aseptically added to 400 mL sterile medium in a 600-mL jacketed glass bioreactor to reach an OD_{620nm} of 0.7. The temperature was controlled at 32 ˚C using a circulating water bath (Haake Gebruder, Berlin, Germany). Anaerobiosis was maintained by bubbling nitrogen at the beginning of the experiment, and then a nitrogen bag was connected to the head plate. The pH was measured using a pH electrode connected to a pH control unit (Inventron AB, Mölndal, Sweden) to control a peristaltic pump (Alitea, Uttran, Sweden) for addition of 5N NH_4OH. Samples were collected and checked for cell growth and concentrations of substrate and metabolites.

2.4. Bioreactor design, preparation of immobilization matrix and cell immobilization

The bioreactor was composed of two main units: the packed bed column and the reactor vessel connected together via autoclavable tygon tubing (Figure 1). Both units were water-jacketed for temperature control at 32 ˚C using a circulating water bath. The reactor vessel was equipped with a stirring device and a head plate with ports for pH electrode, base addition,

nitrogen gas bag connection, nitrogen bubbling, and sampling. The maintenance of environmental conditions was done as described in Section 2.3 and shown in Figure 1.

The immobilization matrices coated with PEI were prepared as described elsewhere (Senthuran et al., 1997). Dried Luffa fruit was initially cut into small pieces (25-30 mm length x ~5 mm diameter). Poraver beads and the cut Luffa (Supplementary Figure S1) were washed thoroughly with distilled water and dried at 105 ˚C. The matrices were resuspended in 2% (w/v) aqueous solution of PEI, pH 7 and autoclaved for 20 min at 120 ˚C. Subsequently, the matrices were washed and dried at 50 ˚C for about 12 h, and packed (50 g each) into the column (20 cm height x 5 cm internal diameter) and autoclaved again with the fermentation medium (1/4 filled).

For cell immobilization, the reactor vessel containing 300 mL medium was inoculated with 15 mL of freshly prepared culture. The temperature was controlled at 32 ˚C and pH at 6.5 using 5 N NH_4OH and fermentation was continued for 3 days in the reactor vessel only, until the OD_{620nm} reached ~10. One hundred milliliters of fresh medium was added and the whole culture was recirculated over the packed bed column and back for 48 h at a rate of 15 $mL.min^{-1}$ using a peristaltic pump (Alitea, Uttran, Sweden). At the end of the immobilization cycle, spent broth was removed and a new immobilization cycle was initiated by aseptic addition of 100 mL medium to the packed bed column to prevent drying of the matrix and cells, and 300 mL to the reactor vessel. The latter was inoculated with 15 mL of fresh culture and the cells were allowed to grow under the same environmental conditions as the first immobilization cycle without circulation until OD_{620nm} of 10 was reached, and subsequently the broth was recirculated through the packed bed column for 2 days. The steps for immobilization were repeated for 3-5 cycles to build up the cell density.

2.5. Repeated recycle-batch fermentation using immobilized cells

After cell immobilization, free and weakly adsorbed cells were removed by recirculation of 900 mL of sterile saline solution (3 runs, 300 mL each) through the bioreactor at a rate of 15 $mL.min^{-1}$. Subsequently, 400 mL of the fresh medium was added to the reactor vessel and recirculated through the packed bed column to start the fermentation, which was continued under the conditions described in Section 2.3 until complete consumption of glycerol. Samples were collected from the reactor vessel at regular time intervals for analysis; the first sample was collected after recirculation of the medium to the packed bed column for 15 min (PEI-Poraver)-30 min (PEI-Luffa) due to the time required for loading the column with the fresh medium and achieving medium homogeneity throughout the whole bioreactor. The steps of washing with saline solution, medium exchange and fermentation were repeated for several consecutive runs.

2.6. Continuous production of propionic acid

The continuous fermentation was done using both free cells and immobilized cells. The cells were immobilized on 200 g PEI-treated Poraver in the packed bed column (40 cm height x 6.5 cm internal diameter) as described in section 2.4. The medium was continuously circulated between the packed bed column and the 600-ml vessel at a rate of 30 $mL.min^{-1}$. The medium entered the column from the bottom as well as from the side (around the middle of the column) to avoid a severe pH drop in the column as a result of product formation.

The continuous fermentation was preceded by batch (free cells) or recycle-batch fermentation (immobilized cells) using a medium volume of 600 mL, and the two reactors were run in parallel. Three dilution rates were tested consecutively (0.057, 0.075 and 0.1 h^{-1}) each for at least 5 retention times under the fermentation conditions described in Section 2.3. For the batch and the first dilution rate, the medium composition was similar as mentioned in Section 2.2. For the latter two dilution rates, the glycerol and yeast extract concentrations were decreased to 30 and 7.5 $g.L^{-1}$, respectively, in order to decrease medium losses in the effluent stream.

2.7. Analytical procedures

Cell growth was monitored by measuring OD at 620 nm using an Ultrospec 1000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and correlating it with cell dry weight (CDW), which was determined by centrifugation of 10 mL fermentation broth at 4 000 xg for 20 min in a dried preweighed tube and drying the cell pellet for 12 h at 105 ˚C before weighing again.

For determination of the dry weight of the immobilized cells, the whole content of the immobilization column at the end of the repeated recycle-batches was emptied in a pre-dried glass plate, washed with distilled water to remove weakly adsorbed cells and dried at 105 ˚C for 12 h. The increase in the dry matrix weight as a result of cell immobilization was determined (g_{CDW} per 50 g matrix) and the concentration of the immobilized cell dry weight ($g_{CDW}.L^{-1}$ fermentation medium) was calculated.

Glycerol, propionic acid, acetic acid, succinic acid (SA), and n-propanol (n-POH) concentrations were determined by an HPLC instrument (JASCO, Tokyo, Japan) equipped with an RI detector (ERC inc., Kawaguchi, Japan) and a JASCO intelligent autosampler. Separation of the compounds was done on an Aminex HPX-87H chromatography column connected to a guard column (Biorad, Richmond, CA, USA). The column temperature was maintained at 55˚C with the help of a column oven (Shimadzu, Tokyo, Japan). Samples from the bioreactor were diluted with Millipore quality water and mixed with 20% v/v sulphuric acid (20 $\mu L.mL^{-1}$ sample) and then filtered through a 0.45 µm polypropylene filter. Fifty microliter of the sample was injected into the 5-mM H_2SO_4 mobile phase flowing at a rate of 0.6 $mL.min^{-1}$.

The results shown are the mean of analyses performed in duplicates for all the fermentations. In case of experiments using free cells, the provided data are the mean of two independent replicates.

The volumetric productivity (Q_p) and product yield ($Y_{P/S}$) for batch modes of operation were calculated by taking into account the dilution of the medium as a result of base addition as follows:

$Q_p = [(PA_{final} * \text{dilution factor}) - PA_{initial}] / [\Delta t]$

$Y_{P/S} = [(Product_{final} * \text{dilution factor}) - Product_{initial}] / [(Substrate_{final} * \text{dilution factor}) - Substrate_{initial}]$

3. Results and discussion

P. acidipropionici was chosen for propionic acid fermentation based on earlier reports and our preliminary investigations that showed the organism to provide the highest conversion yield and production rate from glycerol among the *Propionibacteria spp.* investigated (Barbirato et al., 1997; Himmi et al., 2000; unpublished data). Glycerol is a more reduced carbon source than glucose, lactose and lactate (VanBriesen, 2002). Theoretically, its metabolism yields 1 mole propionic acid per mole of glycerol (Rogers et al., 2006); however, under experimental conditions, the yield ranges from 0.6 to 0.9 $mol.mol^{-1}$. Succinic acid, acetic acid and n-propanol are the major by-products (Barbirato et al., 1997). Despite the low yield, glycerol is still superior to other carbon sources that result in high amounts of acetic acid and lower propionic acid yields.

3.1. Batch fermentation of glycerol with free cells

Batch fermentation of glycerol at pH 6.5 using free *P. acidipropionici* cells is illustrated in Figure 2. Glycerol (42 $g.L^{-1}$) was entirely consumed in 62.5 h yielding 19.5 $g.L^{-1}$ propionic acid (0.64 $mol_{PA}.mol_{Gly}^{-1}$) at a rate of 0.34 $g.L^{-1}.h^{-1}$. Total organic acids yield from glycerol was 75 mol%, 85% of which was propionic acid.

As seen in Figure 2, the free cell fermentation was characterized by a very long lag phase of about 24 h, during which the cell density increased by a factor of 4, and glycerol consumption- and propionic acid production- rates were as low as 0.18 and 0.07 $g.L^{-1}.h^{-1}$, respectively. This

period, accounting for 39% of the fermentation time, explains the low final productivity obtained. During this period, utilization of the rich nutrients in the yeast extract for cell growth as well as synthesis of the enzymes required for glycerol metabolism are expected to delay the onset of glycerol utilization (Barbirato et al., 1997). In the subsequent log phase, the cells grew at the maximum specific growth rate (μ_{max} of 0.10 h^{-1}), and glycerol consumption- and propionic acid production- rates reached 1.27 and 0.64 $g.L^{-1}.h^{-1}$, respectively. Finally when the propionic acid concentration reached 15 $g.L^{-1}$, the growth rate was 0 h^{-1}, but cells were still metabolically active and continued to produce propionic acid at a lower rate.

Increasing the glycerol concentration to 63.6 $g.L^{-1}$, yielded 26.3 $g.L^{-1}$ propionic acid (0.64 $mol_{PA}.mol_{Gly}^{-1}$), and was accompanied by a reduction in the acetic acid, n-propanol and succinic acid yields by 60, 21 and 3%, respectively. A similar behavior has been observed when glycerol concentration was increased from 20 to 70 $g.L^{-1}$ with regards to either acetic acid (Barbirato et al., 1997) or acetic acid and succinic acid (Zhu et al., 2010). Accordingly, the molar propionic acid to total organic acids yield was increased to 89 mol%. The cell growth-, glycerol consumption- and propionic acid production rates for the initial 40 $g.L^{-1}$ glycerol were close to those in the experiment with 40 $g.L^{-1}$ glycerol. However, for the residual 20 $g.L^{-1}$ glycerol, the corresponding rates were decreased from a maximum of 0.06 h^{-1}, 0.96 $g.L^{-1}.h^{-1}$ and 0.48 $g.L^{-1}.h^{-1}$ to 0 h^{-1}, 0.4 $g.L^{-1}.h^{-1}$ and 0.19 $g.L^{-1}.h^{-1}$, and consequently, the overall propionic acid production rate was decreased to 0.26 $g.L^{-1}.h^{-1}$ (Table 1, Figure 2b). This could be the result of nitrogen/vitamin limitation and inhibitory effects of propionic acid towards the end of the fermentation. This inhibition is more potent at low nitrogen/vitamin source concentration (Quesada-Chanto et al., 1998).

3.2. Cell immobilization

P. acidipropionici cells were immobilized by adsorption on the surface of two matrices, Poraver and Luffa. Both have been studied previously in biofilm reactors (Alvarez et al., 2006;

Bruno-Barcena et al., 2000; Guoqiang et al., 1992; Senthuran et al., 1997). Luffa is characterized by having spongy structure with large void space which lowers the risk of reactor clogging. Poraver, in contrast, has larger surface area available for immobilization with smaller void spaces. Its surface is covered by numerous macropores which further increase the surface area and support cell attachment and settlement (Guoqiang et al., 1992).

Since preliminary attempts at immobilization of *P. acidipropionici* on the above matrices had shown poor adsorption of cells, the matrices were pre-treated with the cationic polymer PEI prior to immobilization. Based on adsorption studies, PEI-treated matrices showed much higher ability to bind *P. acidipropionici* cells than non-treated ones (data not shown). At the operating pH, PEI is well known to adsorb strongly to surfaces bearing negative charges; in case of Poraver, it interacts with the SiO^- ions formed from the dissociation of silanol (SiOH) groups in water (Behrens and Grier, 2001), while in Luffa it could bind to OH, COO^- and other negatively charged groups available on the lignocellulosic matrix. The matrices thus acquire positively charged surface that interact with the negatively charged cell surface leading to stronger adsorption of the cells. Cell immobilization was performed using the procedure described earlier for adsorbing *Lactobacillus casei* on PEI-coated Poraver (Senthuran et al., 1997), with the exception that the cells were grown to high density in the reactor vessel before circulation through the column packed with the matrix. This helped to overcome the inhibitory effect of PEI exerted on cell growth (Guoqiang et al., 1992; Senthuran et al., 1997). As a result, the immobilization period was significantly shortened to 1–2 weeks (as compared to a month using the reported method) before the immobilized cells were able to efficiently produce propionic acid from glycerol.

3.3. Recycle-batch fermentation using cells immobilized on PEI-Luffa

PEI-Luffa was observed to be a good matrix for immobilization of *P. acidipropionici*. The cells formed white biofilms, which allowed further increase in the capacity of the matrix (Supplementary Figure S2).

A total of five consecutively repeated recycle-batch fermentations were run, the first four with 40 $g.L^{-1}$ glycerol and the fifth with 63.2 $g.L^{-1}$. The substrate consumption, metabolite formation and cell growth during fermentation batches number 1 and 5 with these two glycerol concentrations are illustrated in Figure 3. For the first four consecutive recycle-batches, average yield, volumetric productivity and final propionic acid concentration were 0.74±0.03 $mol_{PA}.mol_{GLY}^{-1}$, 0.29±0.04 $g.L^{-1}.h^{-1}$, and 20.09±1.5 $g.L^{-1}$, respectively indicating a high degree of process stability (Figure 5a). In comparison with free-cell fermentation at a similar glycerol concentration, the propionic acid yield was 15.6% higher and concentrations of succinic acid, acetic acid and n-propanol were decreased by 15, 28 and 36%, respectively. However, even the volumetric production rate was decreased by 15%, which could be a result of low immobilized cell density due to the small surface area available for immobilization on Luffa and the inhibitory effect of PEI.

The free cell density in the reactor vessel represented as OD_{620nm} was decreased from 9.46 and 10.04 in the first two batches to 6.24 and 6.0 for the last batches, suggesting increased specific cell productivity. Increasing the glycerol concentration to 63.2 $g.L^{-1}$ resulted in 50% reduction of the volumetric propionic acid production rate. The concentration of succinic acid and n-propanol was lower than in free-cells fermentation; however, the acetic acid concentration was higher and led to a decreased molar ratio of propionic acid to acetic acid from 38.6 to 31.0 $mol.mol^{-1}$. Owing to the low volumetric productivities achieved, the PEI-Luffa system was not considered to provide economic advantages for the production of propionic acid.

3.4. Recycle-batch fermentation using cells immobilized on PEI-Poraver

Poraver supported the attachment of higher cell density than PEI-Luffa. Propionic acid production using cells adsorbed to PEI-Poraver was investigated for nine consecutively repeated recycle-batches, five with 40 $g.L^{-1}$ of glycerol, 3 with 65 $g.L^{-1}$ and a single batch with 84.6 $g.L^{-1}$. The results of the fermentation for batches 5, 7 and 9 are presented in Table 1 and Figure 4.

Using 40 $g.L^{-1}$ glycerol, the propionic acid production rate reached a maximum of 0.86 $g.L^{-1}.h^{-1}$ in batch 5, which is 10 times higher than that of the first batch. This rate is the highest reported productivity from glycerol using either free or immobilized cells under batch or fed-batch mode of operation. In this batch, 100% glycerol utilization occurred within 25 h, which is 40 and 60 h shorter than the time required for free and PEI-Luffa immobilized cells, respectively (Figure 4a). As a result of the high density of immobilized, adapted cells, the initial phase of slow glycerol consumption observed with free and PEI-Luffa immobilized cells was not observed. The overall glycerol consumption rate was 1.68 $g.L^{-1}.h^{-1}$ for the entire fermentation run and reached a maximum of 2.17 $g.L^{-1}.h^{-1}$ in the initial 13.5 h. The propionate yield was 0.62 $mol.mol^{-1}$ and the molar ratio of propionic acid to total organic acids was constant around 89 mol%.

For the subsequent 3 batches (number 6, 7 and 8) with 65 $g.L^{-1}$ of glycerol, the volumetric productivities were 0.32, 0.43 and 0.42 $g.L^{-1}.h^{-1}$, respectively. A maximum of 28.4 $g.L^{-1}$ propionic acid was obtained in batch 7 (Figure 4b) with a molar percentage conversion and molar ratio to total acids of 63 $mol\%_{Gly}$ and 91 $mol\%_{TA}$, respectively. When the glycerol concentration was increased to 84.6 $g.L^{-1}$, 35.2 $g.L^{-1}$ propionic acid was obtained at a volumetric rate of 0.35 $g.L^{-1}.h^{-1}$. Complete consumption of the glycerol was achieved in 116.5 h. A similar glycerol concentration was either partially fermented (Barbirato et al., 1997) or required 350 h for complete consumption (Zhu et al., 2010) by free-cell batch fermentation. The most significant enhancement was the rapid consumption of glycerol (23.3 $g.L^{-1}$ in 8.25 h) at a rate of 2.8 $g.L^{-1}.h^{-1}$ in the intial stages of fermentation (Figure 4c). The percentage of

propionic acid to total acids was decreased to 83 mol%, caused by elevated formation of succinic and acetic acids.

As seen in Table 1 and Figure 5b, the volumetric productivity decreased by 50% when increasing the glycerol concentration from 42 to 66.6 $g.L^{-1}$; however, the decrease was only 23% upon a further increase to 84.6 $g.L^{-1}$, suggesting increased cell tolerance to propionic acid. Using this bioreactor, the initial slow glycerol consumption phase observed with free cells was not only omitted, but it was turned into the fastest glycerol consumption phase. Also, the increased tolerance to the inhibitory effect of propionic acid allowed conversion of higher glycerol concentrations at high rates. Under the experimental conditions, no clogging of the PEI-Poraver bioreactor was observed. An additional advantage noticed with this type of reactors was the high regenerative ability of the cells even after a period of starvation or exposure to suboptimal conditions (data not shown).

3.5. Continuous production of propionic acid

To avoid the inhibitory effect of propionic acid on cell growth and metabolism, different strategies have been applied including *in situ* product removal (Gu et al., 1999; Jin and Yang, 1998; Wang et al., 2012) and continuous fermentation. The latter allows continuous removal of the produced acid and results in high volumetric productivities; however, one drawback is the washout of the cells at higher dilution rates. This can be avoided by retaining high amounts of cells inside the bioreactor using either an immobilized cell system or a filtration module coupled to the bioreactor for cell recycling (Bories et al., 2004; Boyaval et al., 1994; Lewis and Yang, 1992a). Practically controlling an immobilized cell bioreactor is easier and more economical than using continuous fermentation with cell-recycle.

The PEI-Poraver bioreactor was evaluated for continuous production of propionic acid and compared with the fermentation in continuous stirred-tank bioreactor (CSTR) using free cells (Table 2). The fermentation was started as batch with 40 $g.L^{-1}$ glycerol and subsequently the

system was shifted to a continuous mode. Using free cells, a volumetric productivity of 0.77 $g.L^{-1}.h^{-1}$ was obtained at a dilution rate of 0.057 h^{-1}, with consumption of 18.7 $g.L^{-1}$ of glycerol and production of 13.6 $g.L^{-1}$ propionic acid. Increasing the dilution rate resulted in a reduction in volumetric productivity and yield, and finally cell washout at a rate of 0.1 h^{-1}. When the PEI-Poraver bioreactor was used in a pH-6.5-controlled chemostat, at the lowest feeding rate, 28.3 $g.L^{-1}$ glycerol was consumed giving 14.5 $g.L^{-1}$ propionic acid. The consumed glycerol was decreased when the dilution rate was increased to 0.1 h^{-1} but propionic acid concentration was constant, which resulted in a higher yield of 0.86 $mol_{PA}.mol_{Gly}^{-1}$ and productivity of 1.4 $g.L^{-1}.h^{-1}$. Furthermore, succinic acid, acetic acid, and n-propanol levels were reduced considerably. In this case, percent carbon recovery considering all the fermentation products except the biomass exceeded 100 mol%, indicating that all glycerol was converted to metabolic products while the rich nutrients in the yeast extract were a substrate for cell growth.

3.6. Immobilized cell morphology and density

To further understand the bioreactor performance, free and immobilized *P. acidipropionici* cells were examined using scanning electron microscopy. The free cells were slightly elongated with variable lengths ranging between 1 and 2.5 µm, with distinguished points of cell division (Supplementary Figure S4a). In case of PEI-Luffa, fewer cells were attached to the external surface of the Luffa fibers while more were attached to the fibrous network inside the cut Luffa pieces. PEI-Luffa samples taken at the end of repeated recycle-batch showed alteration in the morphology and size of the cells as the length of some cells increased to ~5 µm (Supplementary Figure S4 b,c). A similar behavior has been reported earlier (Feng et al., 2010; Zhang and Yang, 2009b).This tendency of the cells for elongation explains the lower optical density for the last two batches, as the cells tended to increase in size rather than divide into new cells. It also suggests some kind of physiological adaptation such preferentially metabolize glycerol and, as a consequence, the specific cell productivity and product yield were increased.

A much higher amount of cells were immobilized on PEI-Poraver than on PEI-Luffa (Supplementary Figure S3). The matrix pores (approximately 110 µm internal diameter) were filled with large aggregates of cells bound together and to the immobilization matrix (Supplementary Figure S4 d,e,f), thus explaining the high volumetric productivity. The high degree of cell retention on Poraver could be attributed to the nature of the matrix surface, which is rough and highly porous and provides a larger surface for attachment and shields the cells from being removed by the flowing medium stream. Due to this high density, it was difficult to identify any morphological changes. Upon repeated fermentation, the immobilized cells tended to grow in aggregates rather than as individual cells, which enhanced the amount of immobilized cells.

At the end of the repeated recycle-batch fermentations, determination of the amount of immobilized cells (as dry weight) showed a large difference in the immobilization capacity for the two matrices. The amount of immobilized cells on PEI-Poraver (5.64 g_{CDW}) was 31.3 times higher than that on PEI-Luffa (0.18 g_{CDW}); these cell dry weights were translated to concentrations of 14.1 $g_{CDW}.L^{-1}$ (PEI-Poraver) and 0.45 $g_{CDW}.L^{-1}$ (PEI-Luffa), respectively. Despite the high amount of cells immobilized, the specific cell productivity in case of PEI-Poraver was 14 times lower than that with PEI-Luffa considering only the last recycle-batch in each case, and was 12 times lower than that with free-cell fermentation (63.6 $g.L^{-1}$ glycerol). This could be attributed to the inaccessibility of the cells, trapped inside the biofilm formed on Poraver, to the substrate. On the other hand, the cells on PEI-Luffa seem to exhibit a higher metabolic activity.

4. Conclusion

This study demonstrates the advantages of using immobilized cells for fermentations characterized by product inhibition. It also shows that the choice of the matrix is important for

achieving the desired improvement in fermentation efficiency. In particular, immobilization on PEI-Poraver considerably enhanced propionic acid volumetic production rate. The increased tolerance to propionic acid also allowed faster fermentation of higher glycerol concentrations. The obtained productivities were superior to those reported earlier in either batch or fed-batch modes of operation with equivalent final propionic acid concentration (Table 3).

5. Acknowledgement

The Swedish Governmental Agency for Innovation Systems (Vinnova) is acknowledged for funding the project. Perstorp AB is thanked for coordinating the project.

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Figure captions

Figure 1. Bioreactor design for batch, recycle-batch and continuous production of propionic acid from glycerol showing the two main units: (I) reactor vessel for pH control and sampling, and (II) a packed bed column containing the immobilized cells. In case of immobilized cells, the medium was recirculated between the two units using a peristaltic pump. For continuous fermentation, medium was fed via a pump that also controlled the rate of the outlet product stream.

Figure 2. Batch production of propionic acid from (a) 43 g/L glycerol and (b) 63.6 g/L glycerol using free cells of *P. acidipropionici* DSM 4900 under anaerobic conditions, 32˚C and pH controlled at 6.5 using 5N NH_4OH. Symbols indicate: (♦) glycerol, (■) propionic acid, (▲) succinic acid, ( ) n-propanol and (**x**) acetic acid concentration and cell growth represented as (●) OD 620nm.

Figure 3. Fermentation of glycerol, (a) 43.3 $g.L^{-1}$ and (b) 63.2 $g.L^{-1}$, using *P. acidipropionici* DSM 4900 cells immobilized on PEI-treated Luffa using recycle-batch mode of operation. Symbols indicate: (♦) glycerol, (■) propionic acid, (▲) succinic acid, (**x**) acetic acid and ( ) n-propanol concentrations, and cell growth represented as (●) OD_{620} nm.

Figure 4. Fermentation of (a) 42 $g.L^{-1}$, (b) 66.6 $g.L^{-1}$ and (c) 84.6 $g.L^{-1}$ glycerol using *P. acidipropionici* DSM 4900 cells immobilized on PEI-treated Poraver. Symbols indicate: (♦) glycerol, (■) propionic acid, (▲) succinic acid, (**x**) acetic acid and ( ) n-propanol concentrations, and cell growth represented as (●) OD_{620} nm.

Figure 5. Fermentation kinetics for repeated recycle-batch propionic acid fermentation using immobilized cells on (a) PEI-Luffa and (b) PEI-Poraver , showing propionic acid yield (■), volumetric productivity (▲), *PA/AA* molar ratio (**x**), and concentrations of propionic acid (white bars), and glycerol (grey bars). Experimental details are described in the text.

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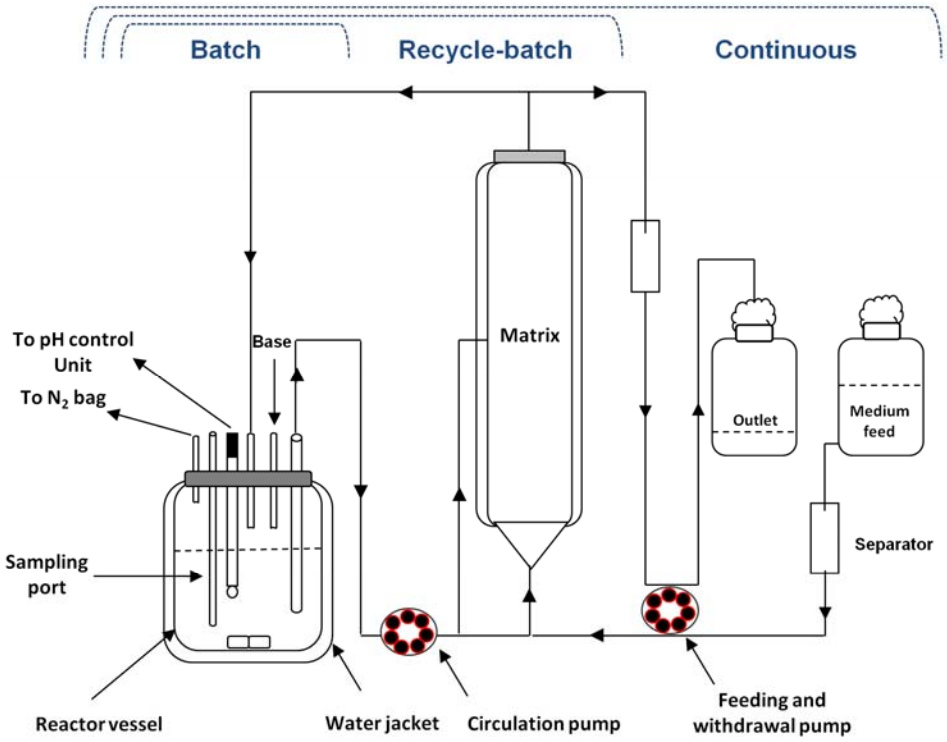

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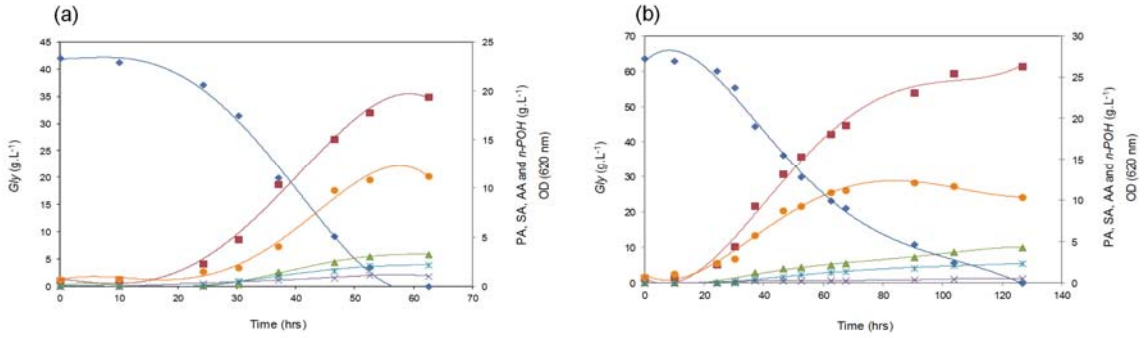

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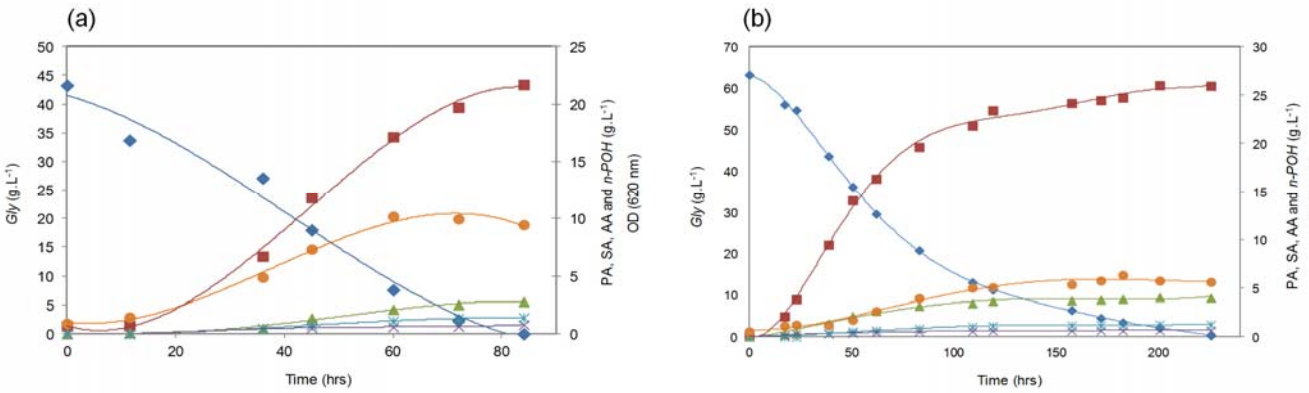

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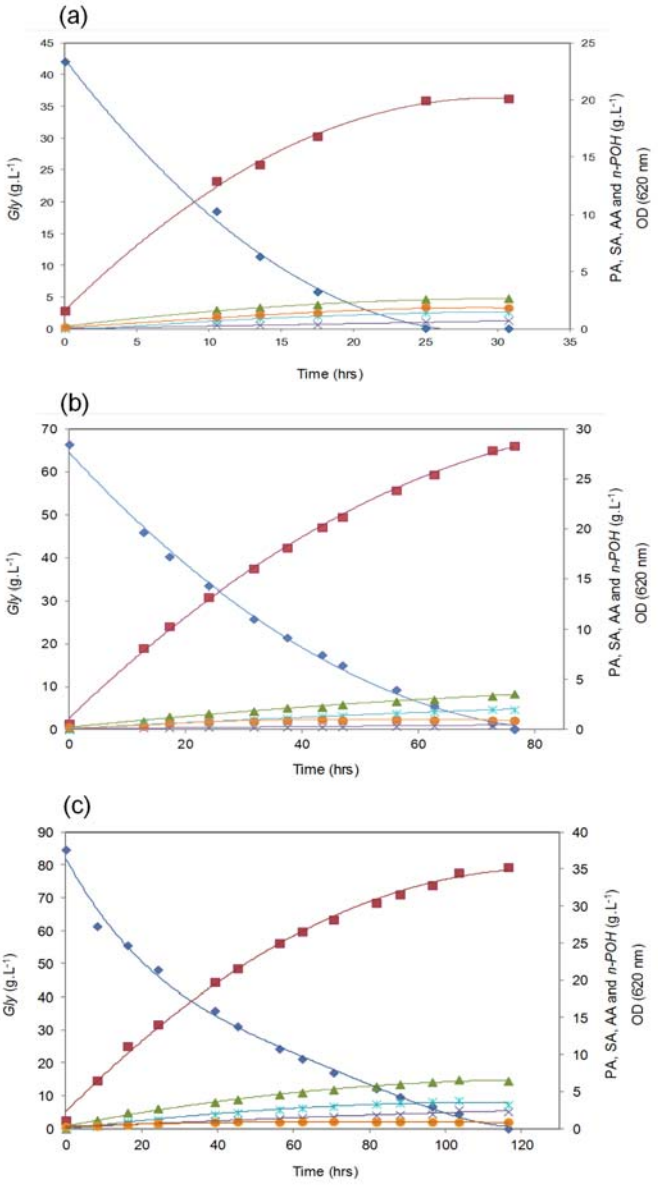

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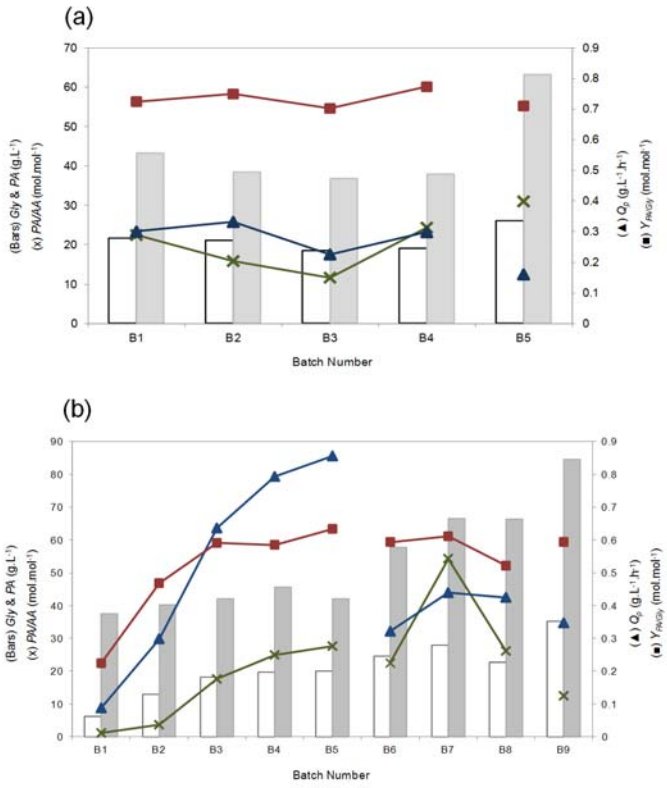

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Table 1

Fermentation data and kinetics for batch production of propionic acid from glycerol using *P. acidipropionici* DSM 4900 free cells and immobilized cells on PEI-Luffa and PEI-Poraver

Bioreactor	Fermentation time (h)	Concentration ($g.L^{-1}$)					OD 620 nm	Q_p ($g.L^{-1}.h^{-1}$)	$Y_{PA/Gly}$ ($mol.mol^{-1}$)
		Gly	PA	SA	AA	n-POH			
Free cells	62.5	42.0±0.50	19.46±0.63	3.25±0.32	1.09±0.10	2.18±0.23	11.88±0.16	0.34	0.64
Free cells	126.75	63.6±0.90	26.31±0.78	4.29±0.38	0.55±0.21	2.35±0.28	10.36±0.05	0.26	0.64
PEI-Luffa (B1)[(a)]	84	43.3±0.01	21.70±0.02	2.77±0.01	0.78±0.00	1.39±0.01	9.46±0.03	0.30	0.72
PEI-Luffa (B5)	225.5	63.2±0.03	26.00±0.02	4.03±0.00	0.68±0.00	1.28±0.01	4.03±0.06	0.16	0.71
PEI-Poraver (B5)	30.75	42±0.01	20.09±0.01	2.65±0.00	0.70±0.00	1.42±0.03	1.80±0.02	0.86	0.64
PEI-Poraver (B7)	76.5	66.6±0.05	28.39±0.02	3.60±0.00	0.51±0.00	2.00±0.01	1.01±0.00	0.43	0.63
PEI-Poraver (B9)	116.5	84.6±0.00	35.23±0.01	6.45±0.00	2.30±0.00	3.22±0.00	0.88±0.02	0.35	0.59

[(a)] (B): Batch number

Gly: Glycerol, PA: Propionic acid, SA: Succinic acid, AA: Acetic acid, n-POH: n-propanol

Q_p: Propionic acid volumetric production rate; $Y_{PA/Gly}$: Propionic acid yield

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Table 2

Fermentation profile and kinetics for continuous production of propionic acid from glycerol using free and immobilized *P. acidipropionici* DSM 4900 cells on PEI-Poraver

Bioreactor	**Free cells**			**Immobilized cells**		
Dilution rate (h^{-1})	0.057	0.075	0.10	0.057	0.075	0.10
Yeast extract ($g.L^{-1}$)	10	7.5	7.5	10	7.5	7.5
Initial glycerol ($g.L^{-1}$)	40	30	30	40	30	30
Final glycerol ($g.L^{-1}$)	22.27±0.13	17.92±0.30	28.53±0.03	10.93±0.03	7.93±0.05	9.23±0.08
Q_p ($g.L^{-1}.h^{-1}$)	0.77	0.47	0.03	0.83	1.12	1.44
$Y_{PA/Gly}$ ($mol.mol^{-1}$)	0.90	0.65	0.22	0.64	0.84	0.86
PA/AA ($mol.mol^{-1}$)	9.53	3.59	0.60	22.13	27.25	34.54
PA ($g.L^{-1}$)	13.59±0.24	6.29±0.09	0.26±0.17	14.53±0.05	14.99±0.02	14.38±0.02
SA ($g.L^{-1}$)	1.07±0.00	0.44±0.03	0.29±0.00	2.61±0.01	2.18±0.05	1.92±0.00
AA ($g.L^{-1}$)	1.16±0.11	1.42±0.01	0.35±0.13	0.53±0.00	0.45±0.00	0.34±0.03
n-POH ($g.L^{-1}$)	1.30±0.15	1.64±0.04	0.29±0.13	1.46±0.00	1.23±0.03	1.09±0.01
OD (620 nm)	13.06±0.04	8.6±0.14	0.8±0.08	10.5±0.02	5.14±0.01	2.56±0.06

PA: Propionic acid, SA: Succinic acid, AA: Acetic acid, n-POH: n-propanol
Q_p: Propionic acid volumetric production rate; $Y_{PA/Gly}$: Propionic acid yield

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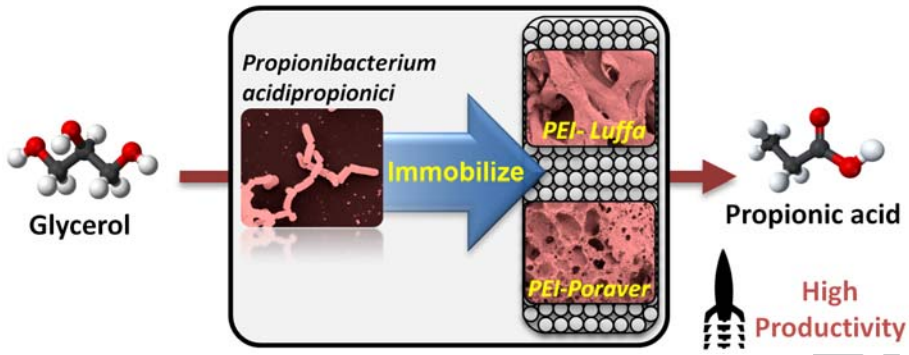
Table 3

Comparison of the results from this study with literature reports on propionic acid fermentation processes with glycerol using free and immobilized cells under batch and fed-batch modes of operation

Microorganism[a]	Total glycerol ($g.L^{-1}$)	Type of immobilization Matrix	Mode of operation	Q_p ($g.L^{-1}.h^{-1}$)	PA ($g.L^{-1}$)	$Y_{PA/Gly}$ ($mol.mol^{-1}$)	Ref
P. acidipropionici ATCC 25562	30	----	Batch	0.24	20	0.83	(Barbirato et al., 1997)
P. thoenii NCDO 1082	102.3[b]	----	Fed-batch	0.15[b]	42	0.51[b]	(Boyaval et al., 1994)
P. acidipropionici ATCC 4875 knockout mutant and adapted culture	~ 40	----	Batch	0.10	26	0.67	(Zhang and Yang, 2009b)
	~ 40	Adsorption on fibrous bed bioreactor (FBR)	Recycle-batch	0.25	23	0.73	
	189.29[b]	Adsorption on FBR	Fed-Batch	0.04[b]	106	0.70	
P. acidipropionici ATCC 25562	20	Entrapment in Ca alginate beads	Recycle-batch	0.8	12	0.75	(Bories et al., 2004)
P. acidipropionici ATCC 25562	20	----	Batch	0.42	12	0.79	(Himmi et al., 2000)
P. acidipropionici ATCC 4965	20	----	Uncontrolled-pH-Batch	0.051	6.77	0.9	(Coral et al., 2008)
P. acidipropionici CGMCC 1.2230	50	----	Batch	0.19	28.53	0.71	(Zhu et al., 2010)
	80	----	Fed-batch	0.2	47.28	0.73	
P. acidipropionici DSM 4900	40 (4 batches)	Adsorption on PEI-Luffa	recycle-batch	0.29	20.09	0.74	This study
	42	Adsorption on PEI-Poraver	recycle-batch	0.86	20.09	0.64	This study
	66.6	Adsorption on PEI-Poraver	recycle-batch	0.43	28.39	0.63	This study
	84.6	Adsorption on PEI-Poraver	recycle-batch	0.35	35.23	0.59	This study

[a] (DSM 4900 = ATCC 25562 = CGMCC 1.2230)
[b] Calculated
PA: propionic acid; Q_p*:* Propionic acid volumetric production rate; $Y_{PA/Gly}$: Propionic acid yield

Graphical abstract



Highlights

- Polyethylenimine-treated matrices for immobilization of *Propionibacteria*
- High propionic acid production rates from glycerol using immobilized cells
- Establishment of stable process for repeated batch production of propionic acid

Batch- and continuous propionic acid production from glycerol using free and immobilized cells of *Propionibacterium acidipropionici*

Tarek Dishisha[a*], Maria Teresa Alvarez[a,b] and Rajni Hatti-Kaul[a]

Figure S1. Different matrices used for immobilization of *Propionibacterium acidipropionici* DSM 4900 cells for propionic acid production. (a) Poraver and (b) cut Luffa pieces.

(a)

(b)

Figure S2. *Propionibacterium acidipropionici* DSM 4900 cells immobilization on PEI-treated Luffa, showing immobilization column packed with PEI-luffa pieces (a) during immobilization step and (b) during propionic acid production. White aggregates of microbial biofilm attached to Luffa matrix could be seen in the latter.

(a) **(b)**

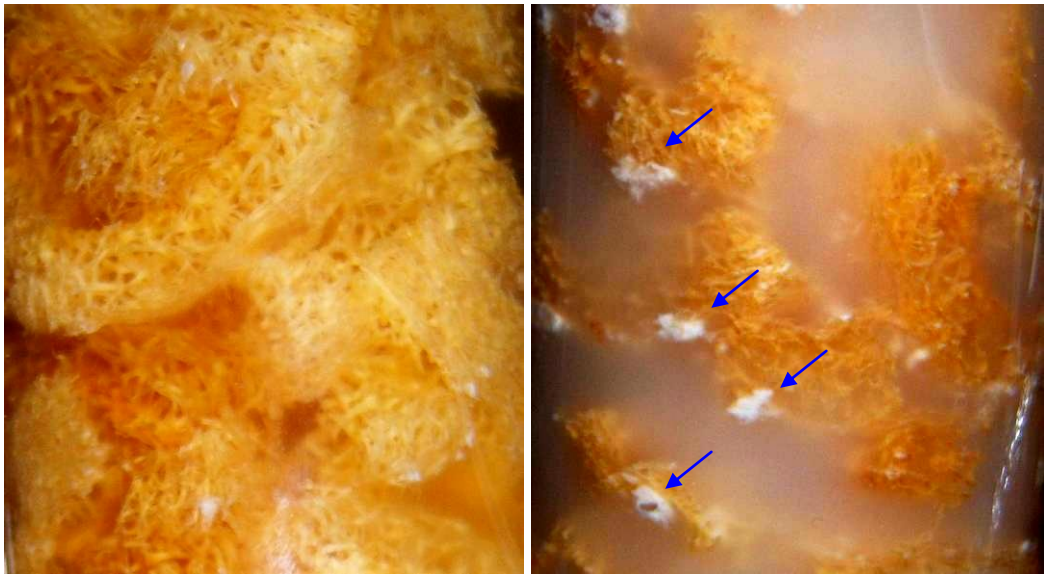


Figure S3. Packed-bed column with PEI-treated Poraver showing the immobilization matrix with immobilized cells of *P*. *acidipropionici* DSM 4900 during propionic acid production from glycerol.

Figure S4. Scanning electron microscopy of *P*. *acidipropionici* DSM 4900 cells growing on glycerol: (a) free cells, (b) Luffa matrix showing fibrous structure with large void volume, (c) cells immobilized on PEI-treated Luffa showing elongated cells, (d) Poraver matrix showing surface structure and pores, (e) a single pore on Poraver showing immobilized *P*. *acidipropionici* cells, and (f) close view to the PEI-Poraver immobilized cells showing the high cell density inside pores.