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Improved propionic acid production from glycerol: combining cyclic batch and sequential batch fermentations with optimal nutrient composition

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Abstract

Propionic acid was produced from glycerol using *Propionibacterium acidipropionici*. In this study, the impact of the concentrations of carbon and nitrogen sources, and of different modes of high cell density fermentations on process kinetics and -efficiency was investigated. Three-way ANOVA analysis and batch cultivations at varying C/N ratios at pH 6.5 revealed that propionic acid production rate is significantly influenced by yeast extract concentration. Glycerol to yeast extract ratio (ww^{-1}) of 3:1 was required for complete glycerol consumption, while maintaining the volumetric productivity. Using this optimum C/N ratio for propionic acid production in cyclic batch fermentation gave propionate yield upto 93 mol% and productivity of 0.53 $gL^{-1}h^{-1}$. Moreover, sequential batch fermentation with cell recycling resulted in production rates exceeding 1 $gL^{-1}h^{-1}$ at initial glycerol upto 120 gL^{-1}, and a maximum of 1.63 $gL^{-1}h^{-1}$ from 90 gL^{-1} glycerol.

Keywords

Propionic acid –*Propionibacterium acidipropionici* – Carbon:nitrogen ratio – High cell density – Cyclic batch fermentation – Sequential batch fermentation

1. Introduction

Propionic acid (PA) is a bulk chemical with an annual production capacity of 349,000 tonnes in 2006 (TranTech-Consultants, 2007). It is widely used as a preservative and is also used as ingredient in animal feed, plastics, herbicides, pharmaceuticals and perfumes (Boyaval and Corre, 1995; Kirschner, 2009; Rogers et al., 2006). Current industrial production of propionic acid is via chemical synthesis from fossil-based raw materials, mainly by oxo-synthesis route from ethylene. Its production by microbial fermentation from renewable resources has attracted increasing attention (Kirschner, 2009; Rogers et al., 2006; TranTech-Consultants, 2007). Different microorganisms can produce propionic acid as a metabolic end product, of which *Propionibacteria* have been the most investigated. These microorganisms can metabolize different carbon sources into propionate through succinate (SA) as intermediate in a so-called dicarboxylic acid pathway (Playne, 1985). Glycerol (Gly) is a more reduced carbon source as compared to sugars and lactate, and induces a homopropionate fermentation behavior generating propionate at high yield with less acetate (AA) as by-product (Barbirato et al., 1997; Bories et al., 2004; Coral et al., 2008; Dishisha et al., 2012; Dishisha et al., 2013; Himmi et al., 2000). Nevertheless, this production route is limited by the strong product-mediated inhibition on cell growth and metabolic activity (Blanc and Goma, 1987). This effect is caused by penetration of the undissociated propionic acid molecules from the solution through the cell membrane leading to disruption of the intracellular buffering system and cellular activities. The excretion of the generated protons is an ATP-dependent step. As a consequence, cell growth and metabolic activity is inhibited due to long term depletion of ATP (Theron and Lues, 2010).

The type and concentration of the nitrogen/vitamin source has earlier been reported to impact the tolerance of *Propionibacteria* to propionic acid (Quesada-Chanto et al., 1998). The availability of a suitable N-source as nutrient elevates the threshold concentration of

propionic acid at which the specific cell growth rate (μ) and specific propionic acid production rate (q_p) become zero (Blanc and Goma, 1987; Obaya et al., 1992; Obaya et al., 1994; Quesada-Chanto et al., 1998). The effect of nitrogen/vitamin source on propionic acid production from glycerol, a carbon source that yields lower cell density than other sugars, has not yet been investigated.

The importance of the nitrogen/vitamin source can also be noticed in batch and fed-batch fermentations. Considerable reduction in propionic acid volumetric production rate has been observed when maintaining the nitrogen/vitamin concentration constant while increasing the concentration of the carbon source in a batch operation and when feeding only a carbon source in the fed-batch mode, respectively (Barbirato et al., 1997; Boyaval and Corre, 1995; Dishisha et al., 2012; Dishisha et al., 2013; Suwannakham and Yang, 2005; Zhang and Yang, 2009a, b). Supplementation of the feeding solution with nitrogen/vitamin source enhanced/maintained the production rates (Ozadali et al., 1996; Paik and Glatz, 1994).

Besides nitrogen source, the process design also plays an important role in determining the economical feasibility of several processes. Propionic acid production was investigated in different process configurations to reach target productivity of 2-3 g L^{-1} h^{-1} required for industrialization (Dishisha et al., 2013; Werpy et al., 2004). High cell density fermentation through immobilization or cell recycling, and the semi-continuous fermentation were the most promising and resulted in considerable enhancement of propionic acid productivity, -yield and -concentration (Blanc and Goma, 1987; Boyaval and Corre, 1987; Colomban et al., 1993; Dishisha et al., 2012; Dishisha et al., 2013; Suwannakham and Yang, 2005; Woskow and Glatz, 1991; Zhang and Yang, 2009a). However, immobilized cell reactors suffered from mass transfer limitation resulting in lower specific cell productivity (Dishisha et al., 2012).

In the present study, the impact of the concentration of glycerol as a carbon source and yeast extract as a nitrogen/vitamin source on propionic acid production was investigated

using statistical analysis. Subsequently, the fermentation kinetics for a chosen set of concentrations was determined under controlled pH conditions. Finally the optimized medium was used in cyclic batch fermentations (CBF) and sequential batch fermentations with cell recycle (SBF) for enhanced propionic acid production.

2. Materials and Methods

2.1. Materials

Glycerol (99%), ammonium hydroxide solution (28%) and L-cysteine HCl, anyhydrous (98%) were products of Sigma-Aldrich (St Louis, MO, USA). Bacto yeast extract (YE) was procured from Difco (BD laboratories, Detroit, MI, USA) and phosphate buffer salts from Merck (NJ, USA).

2.2. Microorganism and preculture preparation

Propionibacterium acidipropionici DSM 4900 was propagated anaerobically as described earlier (Dishisha et al., 2013), where 1 mL of stock culture in 20% v v^{-1} glycerol was transferred to 20 mL of medium containing per liter: 20 g glycerol, 10 g yeast extract, 2.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 0.25 g L-cysteine HCl, (pH 7) in a 30 mL serum bottle. The culture was incubated for 4 days at 32 ºC and the resulting culture was used to inoculate another 20 mL medium and incubated for 2 days. The same culture medium was used for propionic acid production experiments with the exception that glycerol and yeast extract concentrations were varied.

2.3. Evaluating the effect of yeast extract and glycerol on propionic acid production

2.3.1. Factorial design under uncontrolled pH cultivations

A 3^3 factorial design experiment was performed to determine the effect of yeast extract and glycerol on cell growth and propionic acid production. The dependent variable selected for this study was the propionic acid volumetric productivity, expressed in g L^{-1} h^{-1}, and the

independent variables were the concentrations of yeast extract and glycerol. The range and the levels of these variables are given in **Table S1**. Fermentations were done in 100 mL serum bottles containing 90 mL fermentation medium inoculated with 4.5 mL (5% v v^{-1}) of fresh inoculum. The culture was incubated at 32 ºC and samples were collected every 24 hours and analyzed for cell growth and the concentration of glycerol and propionic acid.

2.3.2. Statistical analysis

Statistica software package (Version 5.0) a product of StatSoft, was used for regression and graphical analysis.

2.3.3. Batch production of propionic acid with controlled pH

Twenty milliliters of fresh inoculum was added to 400 mL fermentation medium in a 600 mL jacketed glass bioreactor. The culture was mixed with a magnetic stirring-device at 200 rpm. Temperature was controlled at 32 ºC using a circulating water bath (Haake, Germany), pH was maintained at 6.5 using a pH-electrode connected to pH controller unit (Inventron, Sweden), which controls a pump for addition of 5N NH_4OH. For maintaining anaerobic conditions, the medium was initially bubbled with nitrogen gas and then the headspace was connected to a nitrogen gas bag. Six different combinations of glycerol and yeast extract were evaluated (40:10, 50:10, 60:10, 90:10, 60:20 and 90:30) (g L^{-1} each).

2.4. Propionic acid production in cyclic batch fermentations (CBF)

The CBF were performed in a 3 L bioreactor (Applikon, Microbial Biobundle, The Netherlands) with 1 L working volume. The stirrer speed was maintained at 200 rpm, pH at 6.5 through addition of 5 N NH_4OH, and temperature at 32 ºC via a heating blanket and a cooling finger. The fermentation was started by addition of 50 mL freshly prepared inoculum (5% v v^{-1}) and was run until the glycerol concentration reached 5 g L^{-1}. The subsequent batch was started by replacing 90% of the fermentation broth with an equal volume of fresh medium. Two parallel experimental setups were performed, each composed of three

consecutive cyclic batches. The concentrations of glycerol and yeast extract (g L^{-1} each) in the media in the first set were 60:20 during the three batches. In the second set, the corresponding concentrations were 60:20 for the first batch, and 90:30 for the second and third batches.

2.5. Propionic acid production in sequential batch fermentation

Fifty milliliter of freshly prepared inoculum was added to 1 L fermentation medium containing per liter 60 g glycerol, 20 g yeast extract, 2.5 g K_2HPO_4, 1.5 KH_2PO_4 and 0.25 g L-cysteine HCl in a 3-L bioreactor (Applikon). After autoclaving, the medium was bubbled with nitrogen gas and then connected to a nitrogen gas bag to keep the overhead space saturated with nitrogen. Fermentation conditions were similar to that described in **Section 2.4**. The fermentation was continued for 72 hours after which the broth was withdrawn and the cells were collected by centrifugation at 15,000*g* for 10 min and 4 ˚C.

The pelleted cells were resuspended in 400 mL medium to a final cell concentration of 11.56 g L^{-1} (dry weight), and fermentation was started and continued till consumption of the entire substrate. The steps of centrifugation and cell resuspension were repeated for subsequent batches. Glycerol:yeast extract concentrations (g L^{-1} each) used were as follows: 60:20 for batches 1 and 2, 90:30 for batches 3 and 4, 120:40 for batches 5 and 6, and 150:50 for batch 7. Each concentration was run twice sequentially as a way to confirm the stability of the obtained results, except for batch 7 where incomplete consumption of glycerol was observed.

2.6. Analytical methods

Cell growth expressed as units of optical density (OD) at 620 nm was measured using UV-Vis Spectrophotometer, Ultrospec 1000 (Pharmacia Biotech, Sweden) and correlated with the cell dry weight (CDW). For determination of the cell dry weight, 1 mL of fermentation broth was centrifuged at 15,000*g* for 2 min in weighed pre-dried tube. The cell pellet was then

dried at 105 ºC for 12 h. The weight difference is equivalent to the cell dry weight per milliliter.

Analyses of glycerol, propionic acid, acetic acid, succinic acid and *n*-propanol (*n*-POH) were done using an HPLC system (Jasco, Tokyo, Japan) equipped with an RI-detector (ERC, Taguchi, Japan) and chromatographic oven (Shimadzu, Tokyo, Japan). The separation was done on Aminex HPX-87H cation exclusion chromatographic column (300 x 7.8 mm and particle size 9 µm) connected to a guard column (BioRad, USA) using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1}. Injection volume was 50 µl and the column temperature was kept at 55 ºC. Samples for HPLC were diluted to the required concentration range and then mixed with 20 µl mL^{-1} of 20% v v^{-1} sulfuric acid.

The volumetric- (Q) and specific (q) rates, and product yield ($Y_{PA/Gly}$) were calculated by taking into account the dilution of the medium as a result of base addition as follows:

Q_{PA} (g L^{-1} h^{-1}) = [(PA_{final}*dilution factor) – PA_{initial}] / [Δt]

q_{PA} (g_{PA} g_{CDW}^{-1} h^{-1}) = Q_{PA} / X, for propionic acid production, and

Q_{Gly} (g L^{-1} h^{-1}) = [(Gly_{final}*dilution factor) – Gly_{initial}] / [Δt]

q_{Gly} (g_{Gly} g_{CDW}^{-1} h^{-1}) = Q_{Gly} / X, for glycerol consumption

$Y_{PA/Gly}$ (g g^{-1}) = [(PA_{final}*dilution factor) –$PA_{initial}$] / [(Gly_{final}*dilution factor) - $Gly_{initial}$]

3. Results and discussion

3.1. Medium optimization without controlling the pH

A three-way ANOVA analysis was performed to determine the effect of yeast extract and glycerol concentrations on propionic acid production in fermentations with uncontrolled pH. The minimum and maximum levels of variables used were 30 to 90 g L^{-1} for glycerol and 10 to 30 g L^{-1} for yeast extract, respectively. Three different concentrations of each component

in 9 combinations were used and the cell growth, propionic acid concentration, volumetric production rate and yield were determined (**Fig. 1**, **Table S1**). The obtained data revealed that the concentration of yeast extract was statistically significant ($P < 0.05$) with respect to propionic acid volumetric production rate. The average maximum specific growth rate (μ_{max}) for the different media combinations was 0.037 ± 0.006 h^{-1} indicating minimal impact of glycerol and yeast extract concentrations on the growth rate.

The impact of yeast extract concentration was higher at 30 g L^{-1} glycerol, where increasing its concentration by 10 g L^{-1} was accompanied by increase in the maximum cell density, propionic acid concentration and its volumetric productivity, by 0.55 g_{CDW} L^{-1}, 1.65 g_{PA} L^{-1} and 0.02 g_{PA} L^{-1} h^{-1}, respectively. At 60 or 90 g L^{-1} glycerol, these rates were increased by 0.37 g_{CDW} L^{-1}, 1.10 g_{PA} L^{-1} and 0.01 g_{PA} L^{-1} h^{-1}, respectively. The maximum volumetric productivity achieved was 0.12 g L^{-1} h^{-1} and the maximum specific growth rate was 0.043 h^{-1}.

3.2. Batch production of propionic acid with controlled pH

Fermentations for the production of organic acids are characterized by a reduction in the pH which inhibits cell growth and product formation (Hsu and Yang, 1991). Consequently, increasing the concentration of glycerol from 30 to 90 g L^{-1} at constant yeast extract concentration with uncontrolled pH had limited effect on fermentation kinetics. In order to obtain the actual kinetics, controlled-pH cultivations were performed for a chosen set of glycerol and yeast extract concentrations (g L^{-1} each) of 40:10, 50:10, 60:10, 90:10, 60:20 and 90:30, respectively. The pH was maintained at 6.5 that is located between the optimum value for growth (pH 7) and that for propionic acid production (pH 6) (Hsu and Yang, 1991).

For the different media compositions, the time course for microbial growth, glycerol consumption and metabolites formation are shown in **Fig. S1** and the fermentation kinetics are summarized in **Table 1**. The specific propionic acid production- (q_p), biomass production-

(q_x) and glycerol consumption- (q_s) rates were calculated for each sample point and plotted versus propionic acid concentration at the same point (**Fig. S2**). This correlation was subsequently used for determination of the critical propionic acid concentrations affecting cell growth and metabolic activity as described elsewhere (Blanc and Goma, 1987; Quesada-Chanto et al., 1998). In all the combinations evaluated, consumption of the entire glycerol was achieved with the exception of glycerol: yeast extract of 90:10 where only 90% of the initial glycerol was consumed in 289 h.

Increasing the initial glycerol concentration from 40 g L^{-1} to 90 g L^{-1}, while maintaining yeast extract at 10 g L^{-1} has led to increase in propionic acid concentration from 19.50 to 32.10 g L^{-1}, and maximum cell density from 4.39 to 7.32 g_{CDW} L^{-1}, respectively. Nevertheless, propionic acid yield was constant around 0.68 mol_{PA} mol_{Gly}^{-1}. The fermentation time was also increased from 137 h (40 g L^{-1} glycerol) to 289 h (90 g L^{-1} glycerol) as a result of strong product inhibition, which resulted in reduction of the corresponding production rate from 0.18 to 0.16 g L^{-1} h^{-1} and incomplete consumption of the supplied glycerol in the latter case. Additionally, the concentration of the by-products was increased and the molar ratio of propionic acid to by-products was decreased.

Increasing yeast extract concentrations while maintaining the glycerol concentration constant resulted in increase in final cell density from ~7.3 g_{CDW} L^{-1} at glycerol: yeast extract of 60:10 and 90:10 to 9.2 g_{CDW} L^{-1} with 60:20 and 11.7 g_{CDW} L^{-1} with 90:30. On the other hand, the maximum specific growth rate was not affected and had an average of 0.116 ± 0.006 h^{-1} (**Table 1**).

Increasing yeast extract concentration from 10 to 30 g L^{-1} at constant initial glycerol concentration of 90 g L^{-1} resulted in elevated propionic acid concentration and yield. The most significant outcome was the reduction in the fermentation time by 140 h, hence doubling the volumetric production rate. The amount of base added to maintain the pH was

also decreased by 27%, probably a result of the increase in the buffering effect of the yeast extract components. The increased yield was accompanied by reduction in succinic acid, acetic acid and *n*-propanol production, which could be explained by the availability of co-factors required for the enzymes catalyzing the last steps in the metabolic pathway. The ratio between PA/AA, PA/SA and PA/*n*-POH reached 27.06 mol_{PA} mol_{AA}^{-1}, 12.58 mol_{PA} mol_{SA}^{-1}, 16.78 mol_{PA} mol_{nPOH}^{-1} at glycerol: yeast extract of 90:30, which are 1.6, 1.6 and 1.9 fold higher than the corresponding ratios obtained at glycerol: yeast extract of 90:10. The highest ratio of propionic acid to by-products was observed when 60:20 of glycerol: yeast extract was used, while the lowest was obtained using nutrient combination of 60:10 or 90:10.

Going from glycerol: yeast extract of 90:10 to 90:30 increased the critical propionic acid concentration inhibiting cell growth from 25 to 32 g L^{-1} (**Fig. S2A**). Additionally, critical propionic acid concentration inhibiting metabolic activity was increased from 30 g_{PA} L^{-1} to ~44 g_{PA} L^{-1} for 90:10 and 90:30 (**Fig. S2B**) indicating reduced propionic acid inhibitory effect on *P. acidipropionici* cells.

The plots of propionic acid volumetric productivity, -yield and -its molar ratio to by-products, as a function of the ratio of glycerol to yeast extract (C/N; g g^{-1}) showed a general increase in these parameters with decrease in the C/N ratio (within the tested range) (**Fig. 2A, 2B**). On the other hand, the maximum specific growth rate (μ_{max}) was not affected, indicating absence of inhibitory or stimulatory effects of the carbon- and nitrogen source (**Fig. 2B**).

Based on these results, a ratio of glycerol to yeast extract (g:g) of 3:1 is considered essential for consumption of the entire glycerol amount with minimal effect on volumetric production rate, and was used in the subsequent studies for propionic acid production using CBF and SBF.

3.3. Cyclic batch propionic acid fermentation using *P. acidipropionici*

CBF is a modified repeated batch culture in which a strategy of replacing 90% of the broth at the end of the fermentation with the same volume of fresh medium is advantageous for removing most of the inhibitory products, and yielding more adapted cells which will ensure faster utilization of the substrate and production of the acid in the subsequent batches. The withdrawn broth can be subjected to downstream processing while the subsequent batch is being operated.

During CBF, the effect of well-controlled conditions gave a clear effect on the fermentation time and productivity. In comparison to the pH-controlled batch cultivation with glycerol and yeast extract concentrations of 60:20, the first cycle in both CBF cultures gave shorter batch time (75 h) and higher propionic acid productivity (0.42 g L^{-1} h^{-1}) (**Table 2**, **Fig. 3A, B**). The concentrations of glycerol and yeast extract (g L^{-1} each) in the second and third cycles were either 60:20 (System 1) or 90:30 (System 2).

The long initial glycerol-independent growth observed in the first cycle was decreased from 24 to 11 h in the second cycle 1#2, which resulted in shorter fermentation time (**Fig. 3A, Table 2**). The volumetric production rate and propionate yield were however close to that in the first batch. The maximum specific growth rate was reduced from 0.106 h^{-1} in the first batch, to 0.073 and 0.027 h^{-1} in cycles 1#2 and 1#3, respectively.

In system 2#, when higher glycerol and yeast extract concentrations were used, the volumetric productivity in cycle 2#2 and 2#3 were increased by 3.4% and 27.5%, respectively. Moreover, the propionate yield reached 0.93 mol_{PA} mol_{Gly}^{-1} in the last cycle. A reduction in μ_{max} was also observed (**Fig. 3B, Table 2**).

3.4. Sequential batch fermentation with cell recycle (SBF)

High cell density fermentations under batch, fed-batch and continuous modes of operations have been reported to enhance propionic acid volumetric productivity and yield considerably (Boyaval and Corre, 1987; Colomban et al., 1993; Dishisha et al., 2013). However, increasing

the initial glycerol concentration was accompanied with substantial reduction in production rate when heat-treated potato juice was used as nitrogen source (Dishisha et al., 2013). In the present study, four different media combinations with the C/N ratio of 3 were tested in 7 sequential batches with cell recycling. Utilization of the entire glycerol amount was achieved at all C/N combinations with the exception of the highest concentration of 150:50, where only 86% of the initial glycerol amount was consumed.

Fig. 4 shows the microbial growth, glycerol consumption and propionic acid production, and the kinetics are summarized in **Table 2**. The average fermentation time for each medium combination after two sequential batches was 26.5 h (60:20), 33.5 h (90:30) and 62 h (120:40) and the average corresponding propionic acid concentrations were 32.0, 46.0 and 58.0 g_{PA} L^{-1}, respectively. Propionic acid yield was highly stable between 0.70 - 0.74 mol_{PA} mol_{Gly}^{-1}. In all the cases the volumetric productivity exceeded 1 g L^{-1} h^{-1} and reached a maximum of 1.63 g L^{-1} h^{-1} in glycerol: yeast extract combination of 90:30.

In the first two batches using 11.56 and 16.09 g_{CDW} L^{-1}, respectively, as initial cell density with glycerol: yeast extract combination of 60:20, the slopes of glycerol consumption and propionic acid production as a function of time were linear indicating the absence of substrate- or product inhibition. Propionic acid was produced at an average rate of 1.28 ± 0.12 g L^{-1} h^{-1}, which is 4.1 times higher than that for the similar medium composition and lower initial biomass concentration (0.09 g_{CDW} L^{-1}). Shifting from batch to batch the initial biomass concentration was increased. At 90 g L^{-1} glycerol the average production rate was 1.55 ± 0.11 g L^{-1} h^{-1}, which is the highest reported productivity from glycerol using batch mode of operation. Slight product inhibition was observed near the end of each batch and resulted in reduction of consumption rate of the residual 45 g L^{-1} glycerol to 2.19 g L^{-1} h^{-1} after a maximum of 4.19 g L^{-1} h^{-1}. As a consequence, propionic acid production rate reached 1.29 g L^{-1} h^{-1} after a maximum of 2.47 g L^{-1} h^{-1}. Increasing glycerol concentration to 120 g L^{-1}

resulted in 29% reduction in production rate, which was still over 1 g L^{-1} h^{-1} and product inhibition was more significant.

With initial glycerol concentration of 150 g L^{-1}, product inhibition was significant and was accompanied by loss of metabolic activity at propionic acid concentration of 60 g L^{-1}. Product yield obtained was however in the same range as with lower glycerol concentrations.

In case of SBF system, comparison with the earlier reported results using heat-treated potato juice as a nitrogen/vitamin source, revealed increase in propionic acid productivity by 2 and 4 fold at glycerol: yeast extract concentrations (g L^{-1} each) of 90:30 and 120:40, respectively (Dishisha et al., 2013). This indicates the significance of the nitrogen source on propionic acid productivity, and also confirms that the lowered propionic acid productivity with heat-treated potato juice could be improved by increasing its concentration or supplementation with additional N-source/co-factors.

Production of propionic acid by Propionibacteria proceeds through the dicarboxylic acid pathway. The different steps beyond pyruvate to propionate through succinate require different co-factors and vitamins for enzymatic activity such as vitamin B12, biotin, and pantothenic acid (Hettinga and Reinbold, 1972a, b, c). The complex nitrogen source constitutes the main supply for these co-factors, and limited or unbalanced supply results in variation in by-products pattern and fermentation kinetics. For instance, 0.5 mg L^{-1} biotin was added to potato juice for enhancing the fermentation kinetics (Dishisha et al., 2013; unpublished data) and supplementation of whey with yeast extract was essential (Colomban et al., 1993). Increasing nitrogen/vitamin source concentration will ensure supply of these co-factors in excess, and hence higher propionic acid production rates and -yields. Also, the amino acids content of the nitrogen source, mainly arginine and aspartic acid act as a buffering system enhancing the propionic acid fermentation kinetics through lowering the inhibitory effect of the acid on the producing cells (Guan et al., 2013).

4. Conclusion

The present study clearly shows that the nitrogen/vitamin source plays an important role in propionic acid production. Modification of the conventional batch fermentation to CBF or SBF involving cell recycle, and operation using optimal nutrient composition and good pH-control, improved the process by shortening the fermentation time and maintaining high propionic acid productivity. It is possible that after optimization of the CBF and SBF processes with the cheap nitrogen source "e.g. heat-treated potato juice (Dishisha et al., 2013)", these strategies could be easy-to-apply for large scale propionic acid bioproduction.

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6. References:

1. Barbirato, F., Chedaille, D., Bories, A., 1997. Propionic acid fermentation from glycerol: Comparison with conventional substrates. Appl. Microbiol. Biotechnol. 47, 441-446.
2. Blanc, P., Goma, G., 1987. Kinetics of inhibition in propionic acid fermentation. Bioprocess Eng. 2, 175-179.
3. Bories, A., Himmi, E., Jauregui, J.J.A., Pelayo-Ortiz, C., Gonzales, V.A., 2004. Glycerol fermentation with *Propionibacteria* and optimisation of the production of propionic acid. Sci. Aliments 24, 121-135.
4. Boyaval, P., Corre, C., 1987. Continuous fermentation of sweet whey permeate for propionic-acid production in a CSTR with Uf recycle. Biotechnol. Lett. 9, 801-806.
5. Boyaval, P., Corre, C., 1995. Production of propionic acid. Lait 75, 453-461.
6. Colomban, A., Roger, L., Boyaval, P., 1993. Production of propionic acid from whey

permeate by sequential fermentation, ultrafiltration, and cell recycling. Biotechnol. Bioeng. 42, 1091-1098.

7. Coral, J., Karp, S.G., Vandenberghe, L.P.D., Parada, J.L., Pandey, A., Soccol, C.R., 2008. Batch fermentation model of propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. Appl. Biochem. Biotechnol. 151, 333-341.
8. Dishisha, T., Alvarez, M.T., Hatti-Kaul R., 2012. Batch- and continuous propionic acid production from glycerol using free and immobilized cells of *Propionibacterium acidipropionici*. Bioresour. Technol. 118, 553-562.
9. Dishisha, T., Ståhl, Å., Lundmark, S., Hatti-Kaul R., 2013. An economical biorefinery process for propionic acid production from glycerol and potato juice using high cell density fermentation. Bioresour. Technol. 135, 504-512.
10. Guan, N., Liu, L., Shin, H.D., Chen, R.R., Zhang, J., Li, J., Du, G., Shi, Z., Chen, J., 2013. Systems-level understanding of how *Propionibacterium acidipropionici* respond to propionic acid stress at the microenvironment levels: Mechanism and application. J. Biotechnol. 167, 56-63.
11. Hettinga, D.H., Reinbold, G.W., 1972a. Propionic acid bacteria – a review .1. Growth. J. Milk Food Technol. 35, 295-301.
12. Hettinga, D.H., Reinbold, G.W., 1972b. Propionic acid bacteria – a review .2. Metabolism. J. Milk Food Technol. 35, 358-372.
13. Hettinga, D.H., Reinbold, G.W., 1972c. Propionic acid bacteria – a review .3. Miscellaneous metabolic activities. J. Milk Food Technol. 35, 436- 447.
14. Himmi, E.H., Bories, A., Boussaid, A., Hassani, L., 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*. Appl. Microbiol. Biotechnol. 53, 435-440.
15. Hsu, S.T., Yang, S.T., 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: Effects of pH. Biotechnol. Bioeng. 38, 571-578.
16. Kirschner, M., 2009. Chemical profile: Propionic acid. In ICIS Chemical Business. Available at: http://www.icis.com/Articles/2009/03/30/9203721/chemical-profile-propionic-acid.html (Last checked 3 November 2014).
17. Obaya, M.C., Ramos, J., Eng, F., Villa, P., Valdes, E., Martinez, A., Gonzalez, J., Berovides, E., Williams, I., Chivas, M., Cuellar, A., 1992. Production of propionic acid by microbiological way .1. Influence of the initial sugars and product concentrations. Acta Biotechnol. 12, 269-276.
18. Obaya, M.C., Ramos, J., Villa, P., Valdes, E., Eng, F., 1994. Production of propionic acid by microbiological way .2. Effect of the pH value on cell growth and acid production. Acta Biotechnol. 14, 45-51.
19. Ozadali, F., Glatz, B.A., Glatz, C.E., 1996. Fed-batch fermentation with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. 44, 710-716.
20. Paik, H.D., Glatz, B.A., 1994. Propionic acid production by immobilized cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl. Microbiol. Biot. 42, 22-27.
21. Playne, M.J., 1985. Propionic and butyric acids. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology: The Principles, Applications and Regulations of

Biotechnology in Industry, Agriculture and Medicine. Oxford: Pergamon, pp. 731-759.

22. Quesada-Chanto, A., Da Costa, J.P.C.L., Silveira, M.M., Schroeder, A.G., Schmid-Meyer, A.C., Jonas, R., 1998. Influence of different vitamin-nitrogen sources on cell growth and propionic acid production from sucrose by *Propionibacterium shermanii*. Acta Biotechnol. 18, 267-274.
23. Rogers, P., Chen, J.-S., Zidwick, M.J., 2006. Organic acid and solvent production. Part II: Propionic and butyric acids and Ethanol. In: M. Dworkin (Ed.), The Prokaryotes: Symbiotic associations, biotechnology, applied microbiology, vol. 1, third ed. Springer, Berlin / Heidelberg, pp. 611-671.
24. Suwannakham, S., Yang, S.T., 2005. Enhanced propionic acid fermentation by *Propionibacterium acidipropionici* mutant obtained by adaptation in a fibrous-bed bioreactor. Biotechnol. Bioeng. 91, 325-337.
25. Theron, M.M., Lues, J.F.R., 2010. Organic acids and food preservation. CRC Press.
26. TranTech-Consultants, 2007. Chemical profile: Propionic acid. In: ICIS Chemical Business. Available at: http://www.icis.com/Articles/2007/10/01/9065938/chemical-profile-propionic-acid.html (Last checked 3 November 2014).
27. Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., White, J., Manheim, A., Elliot, D., Lasure, L., Jones, S., Gerber, M., Ibsen, K., Lumberg, L., Kelley, S., 2004. Top value added chemicals from biomass, Volume 1- Results of screening for potential candidates from sugars and synthesis gas (U.S. Department of Energy, Oak Ridge, TN, August 2004; available at www.eere.energy.gov/biomass/pdfs/35523.pdf) (Last checked 3 November 2014).
28. Woskow, S.A., Glatz, B.A., 1991. Propionic acid production by a propionic acid tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl. Environ. Microb. 57, 2821-2828.
29. Zhang, A., Yang, S.T., 2009a. Engineering *Propionibacterium acidipropionici* for enhanced propionic acid tolerance and fermentation. Biotechnol. Bioeng. 104, 766-773.
30. Zhang, A., Yang, S.T., 2009b. Propionic acid production from glycerol by metabolically engineered *Propionibacterium acidipropionici*. Process Biochem. 44, 1346-1351.

Legends to figures

Fig. 1 Effect of yeast extract and glycerol concentrations on: **A)** maximum specific growth rate of cells (μ_{max}), **B)** maximum OD_{620nm}, **C)** propionic acid concentration, and **D)** propionic acid volumetric production rate in fermentations using *P. acidipropionici*. The concentration of glycerol in the medium was (g L^{-1}): 30 (x), 60 (■), and 90 (▲), respectively. Propionic acid production was done in serum bottles without shaking and with uncontrolled pH.

Fig. 2 Effect of glycerol to yeast extract ratio (g g^{-1}) on: **A)** propionic acid yield (mol_{PA} mol_{gly}^{-1}) (x, dashed line), and propionic acid volumetric productivity (g L^{-1} h^{-1}) (■, solid line), and **B)** molar ratio of propionic acid to by-products (mol_{PA} $mol_{by\text{-}products}^{-1}$) (●, dashed line), and specific growth rate of *Propionibacterium acidipropionici* (▲, solid line). Propionic acid production was performed in a batch mode of operation with controlled pH.

Fig. 3 Production of propionic acid by cyclic batch fermentation (CBF) using *P. acidipropionici* DSM 4900 with recycling 10% of the fermentation broth. The symbols represent the concentrations of glycerol (▲), propionic acid (●), succinic acid (+), *n*-propanol (-) and cell growth as optical density at 620 nm (x). Two experimental sets are shown: **A)** Three cyclic batches using 60 g L^{-1} glycerol and 20 g L^{-1} yeast extract. **B)** Single batch using 60 g L^{-1} glycerol and 20 g L^{-1} yeast extract followed by two batches using 90 g L^{-1} glycerol and 30 g L^{-1} yeast extract.

Fig. 4 Production of propionic acid by sequential batch fermentation (SBF) using *P. acidipropionici* DSM 4900 with cell recycle showing the concentrations of: **A)** glycerol (●) and propionic acid (▲), **B)** succinic acid (■), *n*-propanol (●), and biomass (▲). The initial concentrations of glycerol and yeast extract (g L^{-1}) were 60:20 for batches 1 and 2, 90:30 for batches 3 and 4, 120:40 for batches 5 and 6, and 150:50 for batch 7.

Table 1. Effect of ratio of carbon:nitrogen source on propionic acid fermentation under pH-controlled conditions

Parameters	**Medium composition (C:N) g L^{-1} each**					
	40:10	**50:10**	**60:10**	**90:10**	**60:20**	**90:30**
Q_p (g L^{-1} h^{-1})	0.18	0.19	0.22	0.16	0.31	0.35
Q_s (g L^{-1} h^{-1})	-0.30	-0.32	-0.42	-0.29	-0.50	-0.56
Y (mol mol^{-1})	0.68	0.73	0.66	0.67	0.78	0.77
Y (g g^{-1})	0.55	0.59	0.53	0.54	0.63	0.62
Initial OD	0.17	0.25	0.17	0.26	0.25	0.23
Final OD	10.18	12.83	16.58	12.34	21.99	25.38
Biomass (g_{CDW} L^{-1})[(a)]	4.39	5.86	7.25	7.32	9.15	11.71
Final PA (g L^{-1})	19.50	25.80	26.00	32.10	33.00	43.40
Final AA (g L^{-1})	0.70	0.70	1.30	1.60	0.50	1.30
Final SA (g L^{-1})	3.00	3.50	4.00	6.40	3.10	5.50
Final *n*-POH (g L^{-1})	1.70	1.80	2.80	2.90	1.50	2.10
PA/AA (mol mol^{-1})	25.67	29.88	16.21	16.26	53.50	27.06
PA/SA (mol mol^{-1})	11.65	11.75	10.36	8.00	16.97	12.58
PA/nPOH (mol mol^{-1})	9.32	11.64	7.54	8.99	17.87	16.78
PA/by-products (mol/mol)	4.14	4.89	3.44	3.36	7.48	5.68
μ_{max} (h^{-1})	0.103	0.112	0.109	0.114	0.112	0.114
Fermentation time (h)	137	150	137	288.5[(b)]	121	~150
Base addition (mL)	63	59	70	141	57	103
Ratio C/N	4	5	6	9	3	3

[(a)] Dilution factor considered
[(b)] Incomplete consumption of glycerol
Abbreviations: Q_P – volumetric productivity; Q_S – volumetric consumption rate; *Y* – Yield; *PA* – propionic acid; *AA* – acetic acid; *SA* – succinic acid; *n-POH* – *n*-propanol

Table 2. Cyclic batch fermentation (CBF) and sequential batch fermentation with cell recycle (SBF)

Glycerol ($g\ L^{-1}$)	Yeast extract ($g\ L^{-1}$)	Q_P ($g\ L^{-1}\ h^{-1}$)	Q_S ($g\ L^{-1}\ h^{-1}$)	Y ($mol\ mol^{-1}$)	μ_{max} (h^{-1})	Initial CDW ($g\ L^{-1}$)
Cyclic batch fermentation (CBF) – System 1						
60	20	0.42	-0.73	0.71	0.106	0.11
60	20	0.37	-0.70	0.74	0.073	1.42
60	20	0.43	-0.79	0.64	0.027	1.21
Cyclic batch fermentation (CBF) – System 2						
60	20	0.42	-0.71	0.73	0.106	0.11
90	30	0.43	-0.74	0.61	0.056	1.52
90	30	0.53	-0.71	0.93	0.074	1.75
Sequential batch fermentation (SBF)						
60	20	1.19	-1.99	0.74		11.56
60	20	1.36	-2.43	0.70		16.09
90	30	1.47	-2.67	0.71		20.30
90	30	1.63	-2.77	0.73		26.81
120	40	1.13	-1.94	0.71		28.79
120	40	1.12	-1.94	0.71		31.86
150	50	0.30	-0.50	0.74		31.28

Abbreviations: Q_P – volumetric productivity; Q_S – volumetric consumption rate; Y – Yield; μ_{max} – maximum specific growth rate; CDW – cell dry weight

Figures

Fig. 1

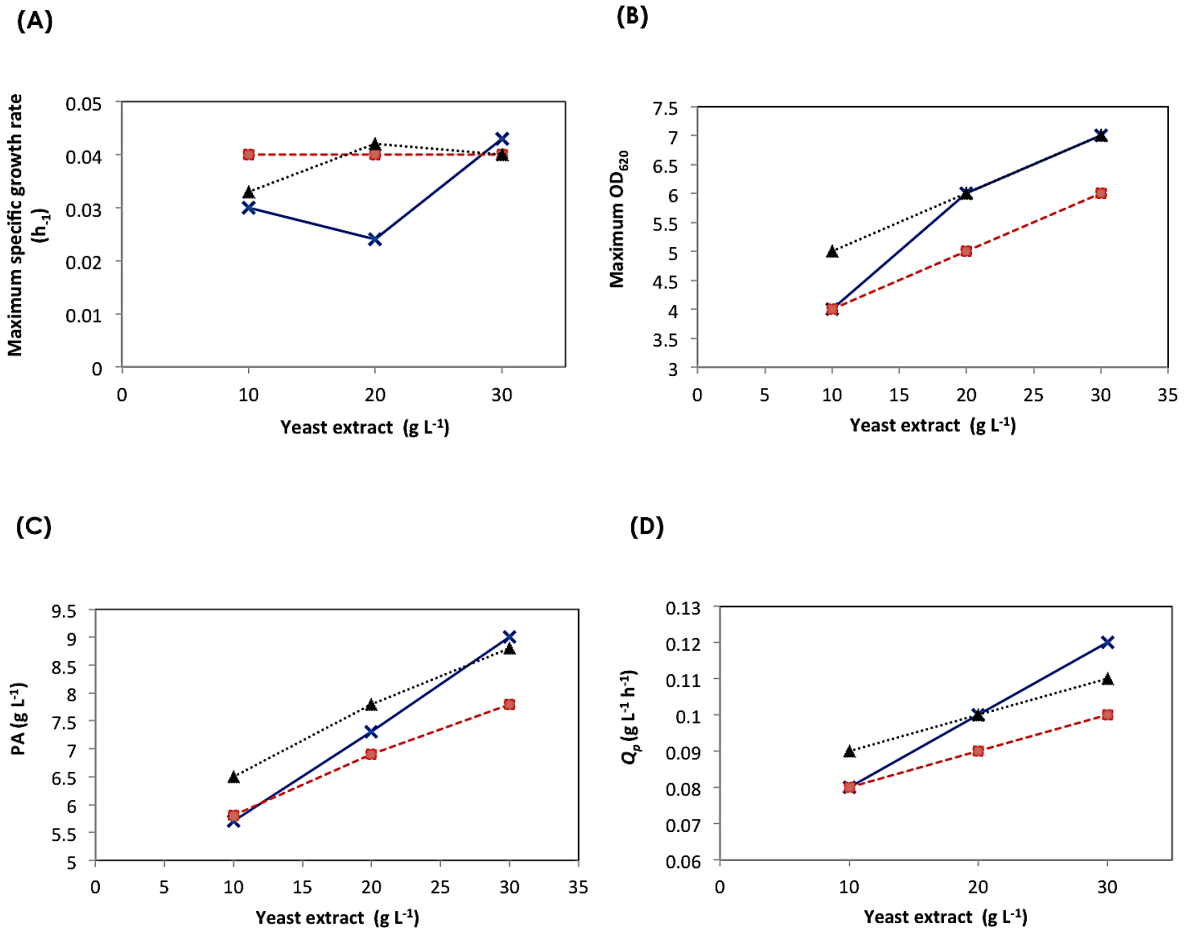


Fig. 2

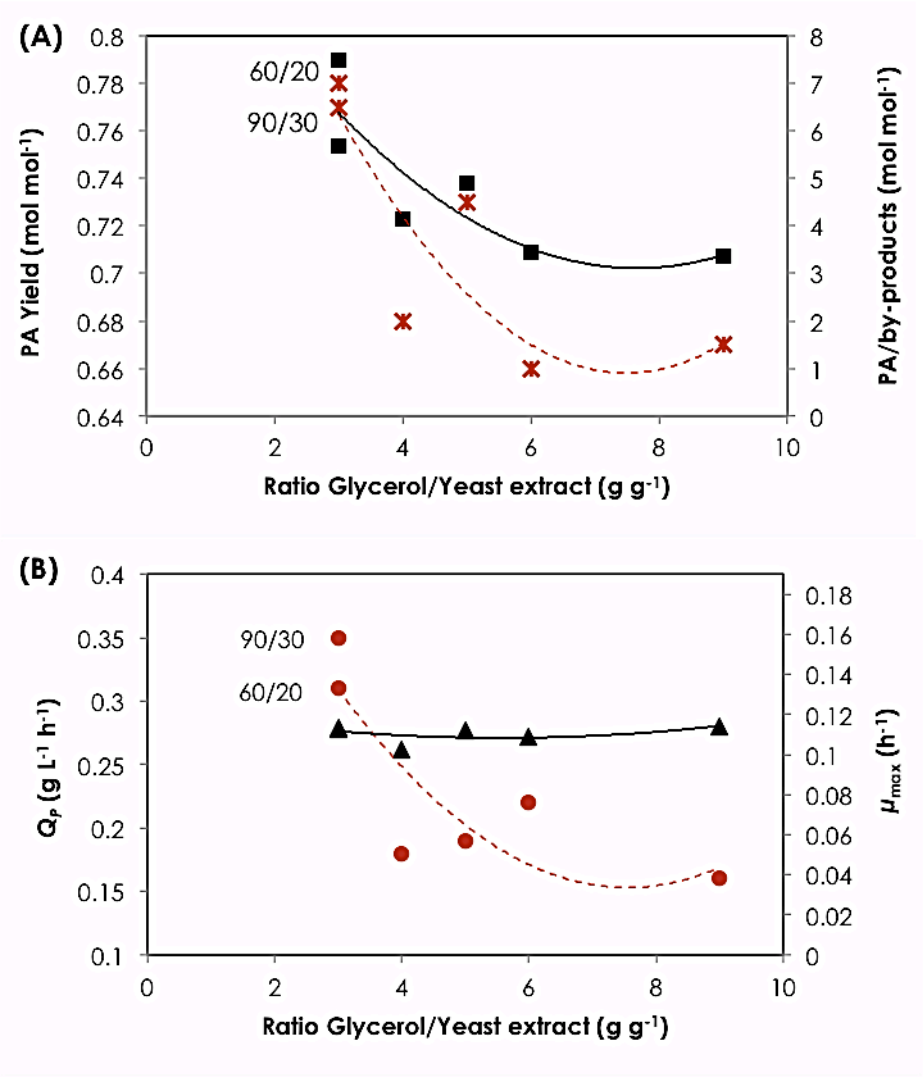

(A)
PA Yield (mol mol-1)
PA/by-products (mol mol-1)
60/20
90/30
Ratio Glycerol/Yeast extract (g g-1)
(B)
Qp (g L-1 h-1)
μmax (h-1)
90/30
60/20
Ratio Glycerol/Yeast extract (g g-1)

Fig. 3

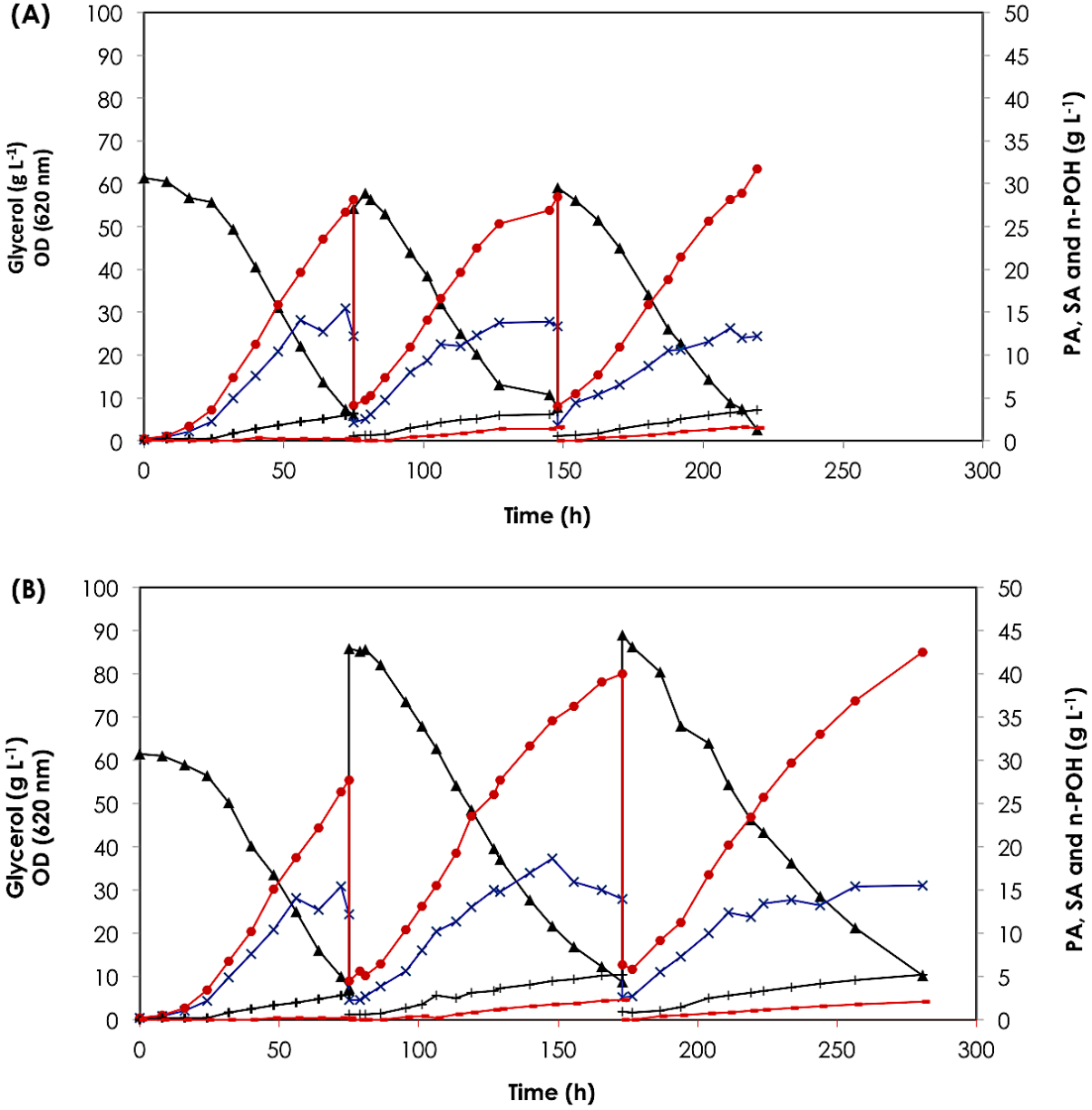
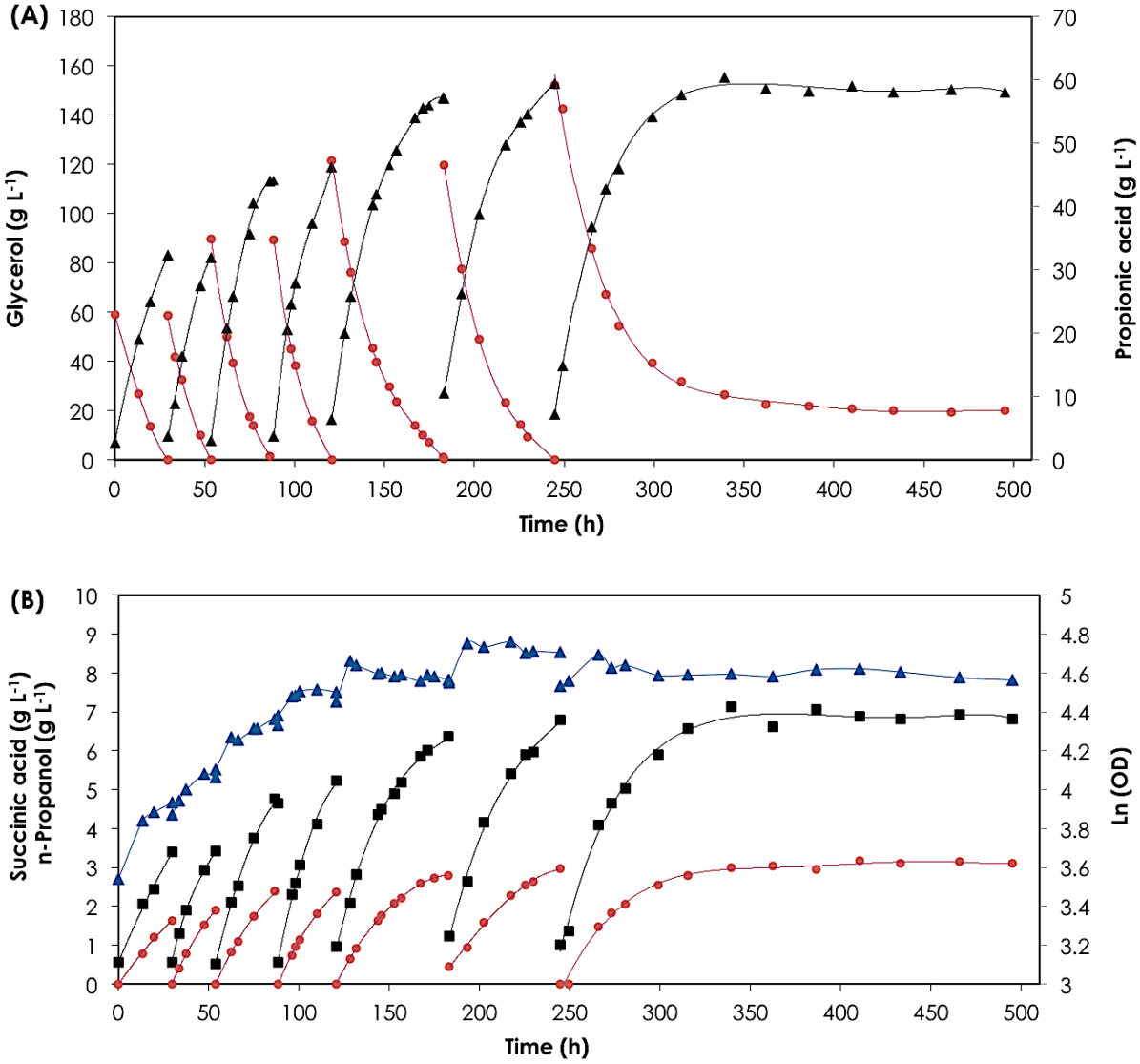

(A)
Glycerol (g L-1)
OD (620 nm)
PA, SA and n-POH (g L-1)
Time (h)
(B)
Glycerol (g L-1)
OD (620 nm)
PA, SA and n-POH (g L-1)
Time (h)

Fig. 4


(A)
Glycerol (g L-1)
Propionic acid (g L-1)
Time (h)
(B)
Succinic acid (g L-1)
n-Propanol (g L-1)
Ln (OD)
Time (h)

Electronic Supplementary Information (ESI)

Improved propionic acid production from glycerol: combining cyclic batch- and sequential batch fermentations with optimal nutrient composition

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Table S1. Effect of different concentrations of yeast extract (YE) and glycerol on cell growth and propionic acid production using *Propionibacterium acidipropionici* DSM 4900 in batch fermentations with uncontrolled culture pH.

Glycerol ($g\ L^{-1}$)	**YE ($g\ L^{-1}$)**	**μ_{max} (h^{-1})**	**Max OD**	**Max propionic acid ($g\ L^{-1}$)**	**Q_p ($g\ L^{-1}\ h^{-1}$)**
30	**10**	0.030	4	5.7	0.08
30	**20**	0.024	6	7.3	0.10
30	**30**	0.043	7	9.0	0.12
60	**10**	0.040	4	5.8	0.08
60	**20**	0.040	5	6.9	0.09
60	**30**	0.040	6	7.8	0.10
90	**10**	0.033	5	6.5	0.09
90	**20**	0.042	6	7.8	0.10
90	**30**	0.040	7	8.8	0.11

Fig. S1 Batch production of propionic acid from glycerol using *Propionibacterium acidipropionici* DSM 4900 with controlled culture pH at 6.5 at varying concentration of glycerol:yeast extract (g L^{-1} each). The symbols represent concentrations of glycerol (■), propionic acid [PA] (▲), succinic acid [SA] (x), acetic acid [AA] (∗), *n*-propanol [n-POH] (●), and cell density represented by OD at 620 nm (♦). Figures represents different combinations of glycerol and yeast extract.

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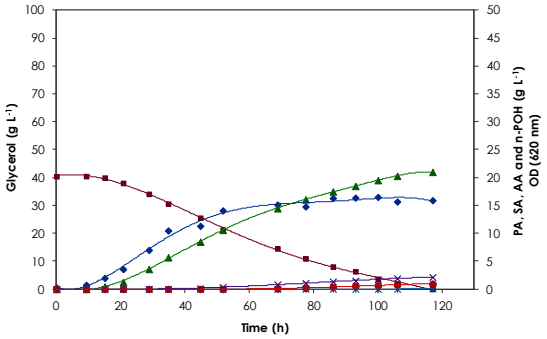

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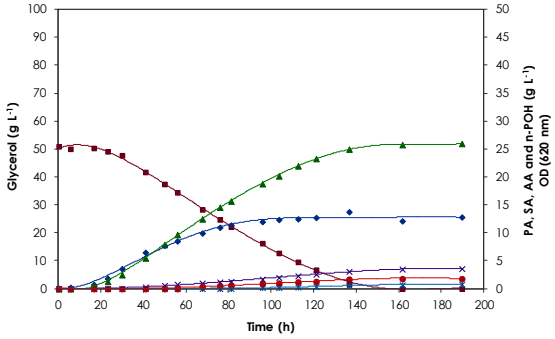

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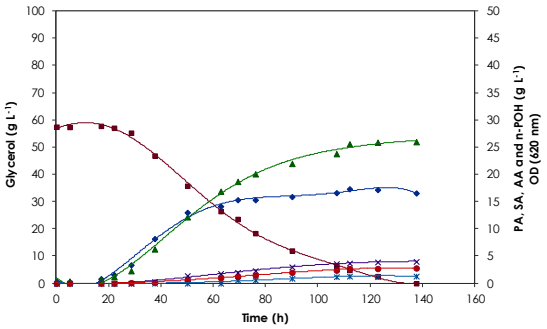

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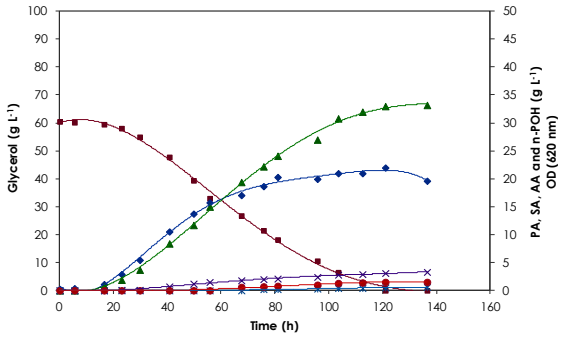

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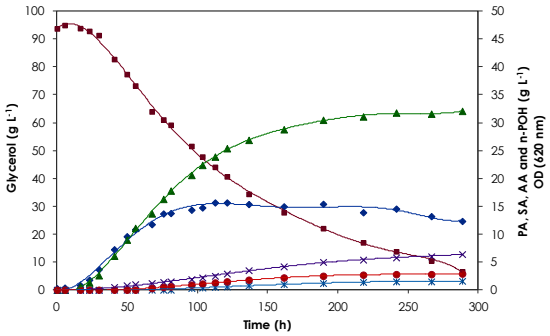

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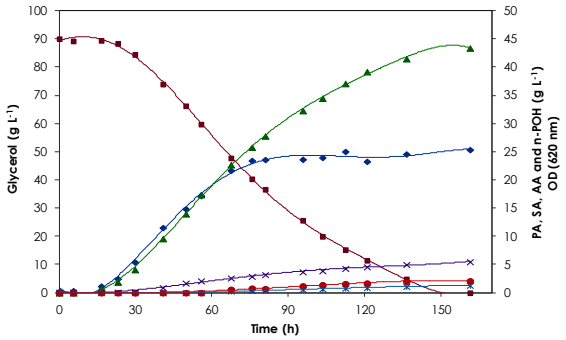
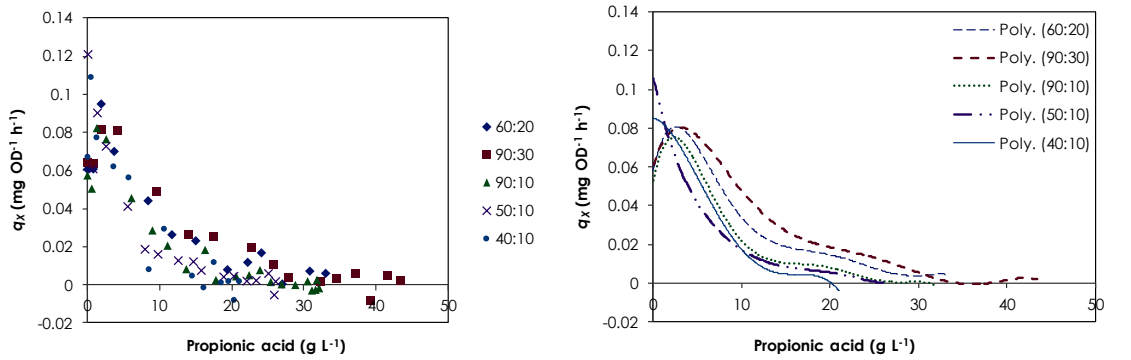


Fig. S2 (A) Specific growth rate (q_X) in h^{-1}, (B) specific propionic acid production rate (q_P) in g L^{-1} h^{-1}, and (C) specific glycerol consumption rate (q_S) in g L^{-1} h^{-1} as function of propionic acid concentration (g L^{-1}). The specific rates were determined for each sampling point of propionic acid fermentation using *Propionibacterium acidipropionici* DSM 4900 growing on different combinations of glycerol and yeast extract (g L^{-1} each).

A



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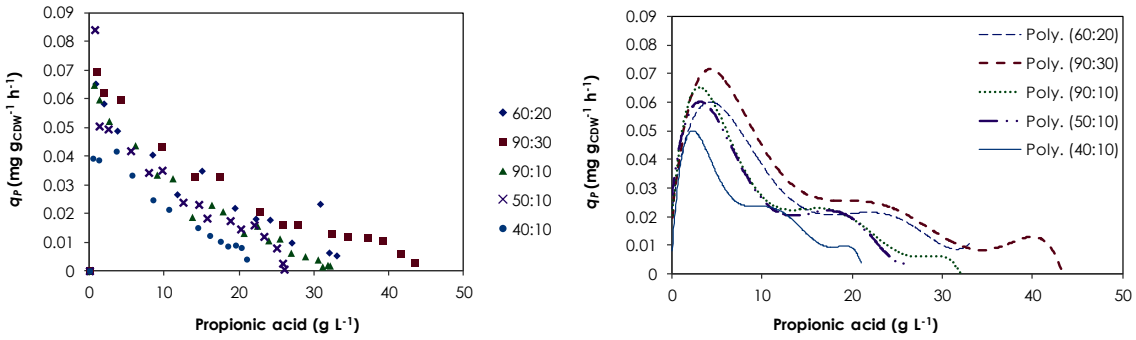
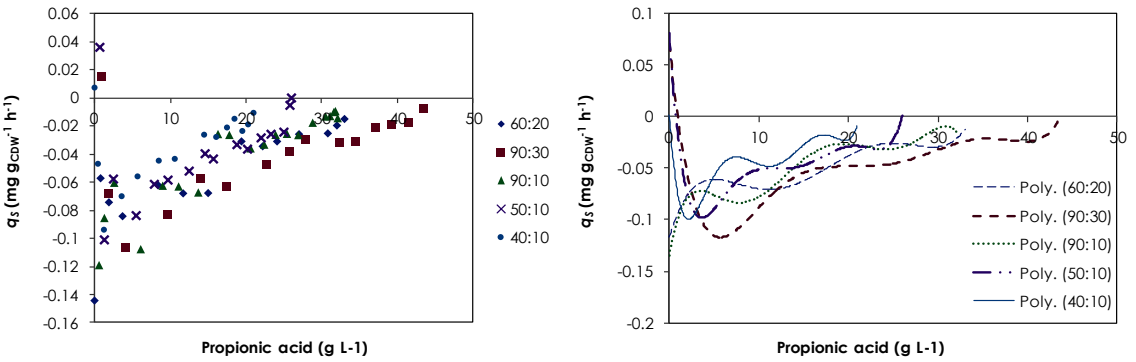
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