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# **An economical biorefinery process for propionic acid production from glycerol and potato juice using high cell density fermentation**

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## Abstract

An economically sustainable process was developed for propionic acid production by fermentation of glycerol using *Propionibacterium acidipropionici* and potato juice, a by-product of starch processing, as a nitrogen/vitamin source. The fermentation was done as high-cell-density sequential batches with cell recycle. Propionic acid production and glycerol consumption rates were dependent on initial biomass concentration, and reached a maximum of 1.42 and 2.30 g L<sup>-1</sup> h<sup>-1</sup>, respectively, from 50 g L<sup>-1</sup> glycerol at initial cell density of 23.7 g<sub>CDW</sub> L<sup>-1</sup>. Halving the concentration of nitrogen/vitamin source resulted in reduction of acetic and succinic acids yields by ~39% each. At glycerol concentrations of 85 and 120 g L<sup>-1</sup>, respectively, 43.8 and 50.8 g L<sup>-1</sup> propionic acid were obtained at a rate of 0.88 and 0.29 g L<sup>-1</sup> h<sup>-1</sup> and yield of 84 and 78 mol%. Succinic acid was 13 g% of propionic acid and could represent a potential co-product covering the cost of nitrogen/vitamin source.

## Keywords:

Biorefinery – Platform chemical – Organic acid – Cell recycle – High cell density fermentation

## 1 Introduction

Propionic acid, a commodity chemical, is used as preservative in feed, foods and pharmaceuticals, and is incorporated in herbicides and polymer industries. Its global production was estimated at 349,000 tons per year in 2006. BASF (Germany and China), Dow Chemical (USA), Eastman Chemical (USA) and Perstorp (Sweden) are the largest producers with annual production capacities of 110, 90, 70 and 50 thousand tons, respectively (TranTech Consultants, 2007). Currently, industrial production of propionic acid utilizes fossil-based resources (Rogers et al., 2006). However, the rising mineral oil prices and its finite nature, increased customer awareness and demand for green products, and increased costs for waste disposal and restrictions on land filling for certain types of waste, has led to increased interest in a more sustainable production of chemicals and materials from renewable bio-based raw materials (Boyaval and Corre, 1995; Ekman and Börjesson, 2011; Thomsen, 2005; Tsoskounogiou et al., 2008). Conversion of bio-based residues or by-products into valuable chemicals offers several potential advantages of low product cost, less environmental impact, less energy requirement and less toxic and biodegradable products. The complexity of the feedstocks however presents a challenge for their use by the chemical industry that has based its production on defined raw materials (Thomsen, 2005).

Production of propionic acid by fermentation, as an alternative to the chemical synthesis, has been investigated earlier using *Propionibacteria*. These microorganisms are able to grow and produce propionic acid using a number of cheap industrial and agricultural by-products. These by-products serve either only as C-source as in case of biodiesel glycerol (Kosmider et al., 2010; Ruhai et al., 2011; Zhang and Yang, 2009) and molasses (Coral et al., 2008; Feng et al., 2011; Quesada-chanto et al., 1994), only as N-source as potato juice (Tatum et al., 1936a), fish hydrolysate (Mahmoud and Levin, 1993) and de-lactose whey (Yang et al., 1995; Yang et al., 1994), or both as C- and N-sources such as corn steep liquor (Ozadali et

al., 1996; Paik and Glatz, 1994), hydrolyzed corn meal (Huang et al., 2002) and whey permeate (Blanc and Goma, 1989; Boyaval and Corre, 1987; Colomban et al., 1993; Yang et al., 1995). Glycerol, in contrast to the other C-sources, gives a higher propionate yield with much less acetic acid as by-product (Barbirato et al., 1997; Dishisha et al., 2012); however, an additional cheap nitrogen/vitamin source for microbial growth and metabolism is required. Potato juice has earlier been reported to have stimulatory effect on the growth of *Propionibacteria* and propionic acid production (Tatum et al., 1936a). Moreover, being a poor C-source, it minimizes acetic acid production, which is formed in high concentrations with other C-source-rich raw materials such as whey permeate.

Industrially, glycerol is obtained as a by-product of fat hydrolysis, ethanol fermentation and biodiesel production process (Agarwal, 1990; Thompson and He, 2006; Yazdani and Gonzalez, 2007). With growing interest in biodiesel as a biofuel over the past few years, glycerol has turned out to be a cheaply available C-source. On the other hand, potato juice is an agro-industrial by-product obtained from potato starch processing. For each ton of potato starch produced, approximately 3.5 tons of potato juice is obtained. This juice is rich in proteinaceous compounds, which are precipitated upon heating of the acidified solution and can be separated, dried and utilized as animal feed. The liquid protein-free fraction, referred to as heat-treated potato juice (HTPJ) is rich in nitrogen containing salts and is currently used as fertilizer (<http://www.starch.dk/isi/starch/tm5www-potato.asp> “Last accessed June 2012”; Ekman and Börjesson, 2011; Tatum et al., 1936a). A life cycle analysis of propionic acid production from glycerol and potato juice has revealed that the fermentation route leads to less carbon dioxide emissions compared to the fossil-based route (Oxo-synthesis), however the energy requirement is higher (Ekman and Börjesson, 2011).

The process of propionic acid fermentation suffers from low volumetric productivity and low final acid concentration due to product inhibition. Retaining high cell density within the

bioreactor by cell immobilization led to enhanced volumetric productivity (Dishisha et al., 2012), however the complexity of controlling such reactors in large scale due to cell growth and pH variation, limits their application for industrial production. The development of high-cell-density fermentation and cell separation techniques has resulted in significant progress in fermentation technology. Several processes for continuous production of propionic acid with cell-recycling have been described, and a volumetric productivity approaching  $14 \text{ g L}^{-1} \text{ h}^{-1}$  was reported (Blanc and Goma, 1989; Boyaval and Corre, 1987; Quesada-chanto et al., 1994). However, the low final acid concentration, the low substrate conversion and the high operating cost are the main obstacles (Boyaval and Corre, 1995).

Sequential batch fermentation with cell-recycle combines the advantages of batch process, which is well understood, easily controlled, and gives high acid concentration, and the continuous mode of operation with cell-recycle that gives high volumetric productivity. Also, the cell separation is done at the end of each batch which reduces operational and maintenance costs considerably. Using this technique, propionic acid was produced from whey permeate with a final concentration of  $35 \text{ g L}^{-1}$  and a volumetric production rate of  $1.2 \text{ g L}^{-1} \text{ h}^{-1}$  (Colomban et al., 1993).

The aim of the present study is to evaluate the feasibility and efficiency of propionic acid production from glycerol with potato juice as a nitrogen/vitamin source, using sequential batch fermentation with cell recycle. The effect of initial biomass concentration on the fermentation kinetics as well as the maximum glycerol concentration consumed under the process conditions used was determined.

## **2 Materials and Methods**

### **2.1 Materials**

Heat-treated potato juice (HTPJ) 3.5-4.5% dry matter content, with composition shown in **Supplementary S1**, was provided by Lyckeby Starch AB, Sweden. It was stored at -20 °C and was thawed just before media preparation. Glycerol (99%), L-cysteine HCl (98%), ammonium hydroxide (28%) and biotin were products of Sigma-Aldrich (St Louis, MO, USA). Phosphate buffer salts were procured from Merck (NJ, USA), while Bacto yeast extract was from Difco (Detroit, Michigan, USA).

## **2.2 Microorganism and culture media**

*Propionibacterium acidipropionici* DSM 4900 was used for propionic acid production. For long-term storage, the culture of the microorganism was maintained in 20% v v<sup>-1</sup> glycerol at -20 °C.

For inoculum preparation yeast extract-based medium, consisting of (per liter) 10 g yeast extract, 20 g glycerol, 2.5 g K<sub>2</sub>HPO<sub>4</sub>, 1.5 g KH<sub>2</sub>PO<sub>4</sub> and 0.25 g cysteine HCl (pH 7), was used. Twenty milliliter medium was placed in a 30-mL serum bottle, boiled, bubbled with nitrogen, and then autoclaved at 121 °C for 15 min. The medium was inoculated from the stock culture (2% v v<sup>-1</sup>) and incubated at 32 °C for 4-5 days and then used to inoculate another 20 mL medium (5% v v<sup>-1</sup>) that was incubated under similar conditions for 2 days (OD<sub>620nm</sub> = 7.3), before transferring the culture to the bioreactor.

The medium for propionic acid production was based on HTPJ (centrifuged for 10 min at 15,000 xg, 4 °C), prepared with variable concentrations of glycerol. After sterilization of the medium by autoclaving at 121 °C for 15 min, sterile-filtered biotin was added to a final concentration of 0.5 mg L<sup>-1</sup>.

## **2.3 Sequential batch propionic acid fermentation with cell recycle**

### 2.3.1 Using similar starting glycerol concentration

A total of 11 consecutive sequential batches with cell recycle were performed in HTPJ medium containing initial glycerol concentration of  $50 \text{ g L}^{-1}$  in all the batches. For the initial 9 batches, HTPJ and biotin concentrations were as described in **Section 2.2**, but for batches 10 and 11, their concentrations were decreased by half. Fermentation in the first batch was started by addition of 20 mL ( $5\% \text{ v v}^{-1}$ ) freshly prepared inoculum to 400 mL HTPJ medium in a 600-mL water-jacketed bioreactor connected to a circulating water bath. The subsequent batches were inoculated using recycled-cells from the preceding batch, after centrifuging the culture broth at  $15,000 \times g$  and  $4^\circ\text{C}$  for 10 min. Consumption of the entire glycerol was chosen as the termination point for each batch. The culture was mixed using a magnetic stirrer at 200 rpm and the temperature was kept at  $32^\circ\text{C}$ . The bioreactor head-plate was equipped with ports for pH electrode, base addition, sampling, bubbling of nitrogen gas, and connection to a nitrogen gas bag. The pH was monitored and controlled at 6.5 through a pH-controller unit (Inventron, Sweden) that controls a peristaltic pump (Alitea, Sweden) for addition of  $5 \text{ N NH}_4\text{OH}$ . Anaerobic conditions were maintained by bubbling the medium initially with nitrogen followed by connecting the bioreactor to a nitrogen gas bag in order to keep the headspace saturated with nitrogen.

### 2.3.2 Using increasing glycerol concentrations

*Production of biomass:* One-liter fermentation medium in a 3 L bioreactor (Applikon, Microbial Biobundle, The Netherlands) was inoculated with 50 mL freshly prepared inoculum. Temperature, pH, stirring and anaerobic conditions were maintained as described above in **Section 2.3.1**. After 3 days, the culture was aseptically centrifuged at  $15,000 \times g$  and  $4^\circ\text{C}$  for 10 min and the cell pellet was used in sequential batch fermentations.

*Sequential batch production of propionic acid:* Cells from biomass production step were



resuspended in 400 mL fresh HTPJ based-medium in 600-mL bioreactor to a final OD<sub>620nm</sub> of 24 and fermentation started. Bioreactor design and operating parameters were as described in **Section 2.3.1**. Five consecutive batches with cell recycle were performed using initial glycerol concentrations of 50, 50, 85, 85 and 120 g L<sup>-1</sup>, respectively.

## 2.4 Analytical methods

*1 Cell growth:* Cell growth was monitored by measuring OD at 620 nm using a spectrophotometer (Ultrospec 1000, Pharmacia Biotech, Uppsala, Sweden) and then correlated with cell dry weight (CDW). The CDW was determined by centrifugation of 1 mL fermentation broth at 15,000 xg for 10 min in a dried preweighed 1.5 mL tube and drying the cell pellet overnight at 105 °C. The increase in weight of the tube equals the cell dry weight per milliliter.

*2 Measurement of substrates and products:* Glycerol, propionic acid, acetic acid, succinic acid, and *n*-propanol concentrations were determined by HPLC (JASCO, Tokyo, Japan) equipped with an RI detector (ERC, Kawaguchi, Japan) and a JASCO intelligent autosampler. Separation of the compounds was done on Aminex HPX-87H chromatographic column connected to a guard column (Biorad, Richmond, CA, USA). The column temperature was kept at 55 °C using chromatographic oven (Shimadzu, Tokyo, Japan). Samples from the bioreactor were diluted with Millipore quality water and acidified with 20% v v<sup>-1</sup> sulfuric acid (20 μL mL<sup>-1</sup> sample) and then filtered through 0.45 μm polypropylene filter. Fifty microliters of the sample were injected in the mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub> flowing at a rate of 0.6 mL min<sup>-1</sup>. Retention times (min) for the different compounds were 11.4 (succinic acid), 13.2 (glycerol), 14.7 (acetic acid), 17.3 (propionic acid), and 26.4 (*n*-propanol).

The data presented is the mean of analyses performed in duplicates. The volumetric- (*Q*)

and specific ( $r$ ) 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:

$$Q_{PA} \text{ (g L}^{-1} \text{ h}^{-1}\text{)} = [(PA_{\text{final}} * \text{dilution factor}) - PA_{\text{initial}}] / [\Delta t]$$

$$r_{PA} \text{ (g}_{PA} \text{ g}_{CDW}^{-1} \text{ h}^{-1}\text{)} = Q_{PA} / X, \text{ for propionic acid production, and}$$

$$Q_{Gly} \text{ (g L}^{-1} \text{ h}^{-1}\text{)} = [(Gly_{\text{final}} * \text{dilution factor}) - Gly_{\text{initial}}] / [\Delta t]$$

$$r_{Gly} \text{ (g}_{Gly} \text{ g}_{CDW}^{-1} \text{ h}^{-1}\text{)} = Q_{Gly} / X, \text{ for glycerol consumption}$$

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

### 3 Results and discussion

#### 3.1 Choice of neutralizing base and nitrogen source

The global consumption of propionic acid is categorized into animal feed and grain preservation (52%), food preservation (15%), herbicides (16%), and as an ingredient in cellulose acetate plastic (10%). Majority of the propionic acid used in feed and grain preservation is in the form of ammonium salt, which is less corrosive to the equipments. Calcium and sodium salts are mainly used in food products (Kirschner, 2009; TranTech Consultants, 2007). Based on this market categorization and expected fields of market growth, production of propionic acid in the form of ammonium salt was chosen for the present investigation by using ammonium hydroxide for controlling the pH during fermentation. Moreover, *Propionibacteria* can utilize ammonia nitrogen for growth and propionic acid production if essential growth factors are supplied (Tatum et al., 1936b; unpublished data).

Potato juice, a by-product of industrial starch processing and a nitrogen rich source, has earlier been reported to have stimulant effect on *Propionibacteria* growth and propionic acid

production, which has been attributed to the presence of ammonium nitrogen in combination with other factors (Tatum et al., 1936b). HTPJ was only recently utilized for promoting the anaerobic digestion process for biogas production (Fang et al., 2011).

### **3.2 Sequential batch production of propionic acid using same initial glycerol concentrations**

Sequential batch fermentation of 50 g L<sup>-1</sup> glycerol using *P. acidipropionici* was investigated for 11 consecutive batches with cell recycle. **Fig. 1** shows the glycerol consumption, metabolites production and cell growth during each batch. The first batch started with 5% v v<sup>-1</sup> inoculum and lasted for 153 h, ending with consumption of the entire glycerol amount (**Supplementary S2**). This run represented a typical batch fermentation and was characterized by an initial slow growth phase for 8 h, followed by log phase at a maximum specific growth rate ( $\mu_{\max}$ ) of 0.1 h<sup>-1</sup> between 8 and 26 h, and then a gradual reduction in the growth rate until the cells entered stationary phase after 74 h of fermentation. Although no consumption of glycerol was observed during the initial 26 h, propionic acid concentration reached 1.8 g L<sup>-1</sup>. Subsequently, glycerol consumption started and continued at a constant rate until the end of the fermentation. A concomitant increase in propionic acid production rate was observed and reached a maximum of 0.29 g L<sup>-1</sup> h<sup>-1</sup>. Overall, 26.8 g L<sup>-1</sup> propionic acid was produced at a rate of 0.22 g L<sup>-1</sup> h<sup>-1</sup> and a molar yield of 0.78 mol<sub>PA</sub> mol<sub>Gly</sub><sup>-1</sup>. Succinic acid (6.3 g L<sup>-1</sup>), acetic acid (2.4 g L<sup>-1</sup>) and *n*-propanol (1.3 g L<sup>-1</sup>) were the main by-products detected in the final broth.

During this batch, critical propionic acid concentration at which the growth rate became almost 0 h<sup>-1</sup>, was 14.7 g L<sup>-1</sup>; indicating that nearly 50% of propionic acid was produced in the stationary growth phase. This partially uncoupled acid production from growth, particularly with glycerol, is important in high cell density fermentations, where the cell growth should be

kept at a minimum rate, while keeping the productivities at maximum rates. In contrast, the critical concentration for suppressing the cells' metabolic activity was not attained, as propionic acid production continued until consumption of the entire glycerol amount. However, the production rate was decreased by 46% when propionic acid concentration reached  $24.0 \text{ g L}^{-1}$  (119 h).

In the subsequent 8 batches, the same medium composition was maintained. The concentration of recycled-cells and fermentation rates were increased when moving from batch to batch. The high density of adapted cells triggered glycerol consumption and propionic acid production right at the start of fermentation runs, and also buffered the inhibitory effect of propionic acid, resulting in almost linear rates of glycerol consumption and propionic acid production throughout each run. The initial biomass concentration was increased 215 times during the 9 batches and reached  $21.5 \text{ g}_{\text{CDW}} \text{ L}^{-1}$  in the 9<sup>th</sup> batch compared to  $0.1 \text{ g}_{\text{CDW}} \text{ L}^{-1}$  in the first batch. This was accompanied by an approximately 6-fold increase in propionic acid volumetric productivity, which reached a maximum of  $1.35 \text{ g L}^{-1} \text{ h}^{-1}$ .

In-depth analysis of the fermentation kinetics showed that propionic acid productivity in batches 2 to 7 was increased logarithmically at a rate of  $\sim 0.25 \text{ g L}^{-1} \text{ h}^{-1}$  per batch, indicating a doubling of productivity every 2.77 batches (**Fig. 2A**). In the subsequent runs, the batch time was stable around 21-22 h and hence the productivities were constant around  $1.30\text{--}1.40 \text{ g L}^{-1} \text{ h}^{-1}$ . This provided an easy way for monitoring the fermentation and predicting the end time of each batch to avoid cell starvation, and also demonstrates the high stability and scalability of the process.

### **3.2.1 Effect of nitrogen source concentration**

In order to investigate the effect of nitrogen source concentration on fermentation kinetics and profile of the by-products, the concentrations of HTPJ and biotin were decreased by 50%

in batches 10 and 11. In the former batch, the volumetric productivity was increased to  $1.42 \text{ g L}^{-1} \text{ h}^{-1}$ , which is the highest reported from glycerol in batch or fed-batch fermentations, due to the high initial cell density of  $23.7 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ . The cell density at the end of the fermentation run was however reduced to  $18.7 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ , which led to decrease in production rate to  $1.11 \text{ g L}^{-1} \text{ h}^{-1}$  during the 11<sup>th</sup> batch but on the other hand was accompanied by increase in biomass to  $20.6 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ .

### 3.2.2 Effect of initial biomass concentration

The volumetric rates of propionic acid production- and glycerol consumption showed a linear correlation with initial biomass concentration in all the 11 batches (**Fig. 2B**). Increasing the initial cell density by a factor of 10 resulted in elevated propionic acid volumetric productivity by  $0.19 \text{ g L}^{-1} \text{ h}^{-1}$ , and glycerol consumption rate by  $0.30 \text{ g L}^{-1} \text{ h}^{-1}$ . The maximum initial biomass concentration reached was  $23.7 \text{ g}_{\text{CDW}} \text{ L}^{-1}$  under the experimental conditions, which limited the volumetric productivity to  $1.42 \text{ g L}^{-1} \text{ h}^{-1}$ . At this stage of negligible growth, the cells act as biocatalyst for biotransformation of substrate into products. The average propionic acid concentration for the 11 batches was  $27.3 \pm 1.1 \text{ g L}^{-1}$  ( $0.77 \pm 0.03 \text{ mol}_{\text{PA}} \text{ mol}_{\text{Gly}}^{-1}$ ) and the overall volumetric productivity was  $0.59 \text{ g L}^{-1} \text{ h}^{-1}$ .

### 3.2.3 Relation between cell growth and propionic acid production

The specific rates of propionic acid production ( $r_{\text{PA}}$ ) and glycerol consumption ( $r_{\text{Gly}}$ ) were constant around  $0.04$  and  $0.06 \text{ g g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ , respectively, during the first 4 batches, followed by gradual increase to a maximum of  $0.06$  and  $0.10 \text{ g g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ , respectively (**Fig. 2C**). This was in contrast to what has been reported earlier for propionic acid production from lactose as carbon source, where the specific productivities were decreased at higher cell densities (Colomban et al., 1993).

The relation between cell growth and propionic acid production was studied through plotting the change in the ratio between the concentrations of propionic acid ( $\Delta PA = PA_{final} - PA_{initial}$ ) and biomass ( $\Delta CDW = CDW_{final} - CDW_{initial}$ ) (**Fig. 2D**). A higher ratio indicates the uncoupled acid production from growth, while a negative value is a sign of reduction in biomass concentration within the batch ( $CDW_{final} - CDW_{initial} < 0$ ). For the initial 4 batches, the ratio was constant at  $5.72 \text{ g}_{PA} \text{ g}_{CDW}^{-1}$ , and subsequently increased gradually to reach a maximum of  $79.63 \text{ g}_{PA} \text{ g}_{CDW}^{-1}$  in the 7<sup>th</sup> batch as a result of the small difference between initial and final cell amount which was  $0.41 \text{ g}_{CDW} \text{ L}^{-1}$ . In batch number 10, the ratio was dropped to a negative value as the cell density at the start of the fermentation was higher than at the end. This could be a result of several factors including reduced space available for cell growth, increased culture viscosity accompanied by mass transfer limitation, and other effects related to changes in water content of the cells, and reduction in nitrogen/vitamin concentration (Colomban et al., 1993). In batch 11, although the growth medium was still half-strength, cells start growing again and the ratio increased to  $5.44 \text{ g}_{PA} \text{ g}_{CDW}^{-1}$ , which indicates the regenerative ability of the cells in presence of glycerol, HTPJ and biotin.

Compared to the other carbon sources like glucose, lactose and sucrose, *Propionibacteria* grow slower on glycerol giving a lower final cell density (Barbirato et al., 1997; Himmi et al., 2000; Zhu et al., 2011). Consequently, ~21 days were required to reach the initial biomass concentration required for optimum productivity. Growing the cells under conditions that allow optimum cell growth initially followed by conditions optimum for propionic acid production could significantly reduce the start-up period. On the other hand, this slow growth and uncoupled growth from acid production is advantageous, as the cells could be utilized for longer periods without the need for bleeding. Also the viscosity of the culture broth was much lower than with other sugars, which further facilitates cell separation (unpublished data).

### 3.2.4 Product and by-products profile

Unlike the dependence of the fermentation rates on initial biomass concentration (**Fig. 2B**), yields of the different metabolites were independent and were constant during the initial 9 batches at  $0.78 \pm 0.03 \text{ mol}_{\text{PA}} \text{ mol}_{\text{Gly}}^{-1}$ ,  $0.12 \pm 0.01 \text{ mol}_{\text{SA}} \text{ mol}_{\text{Gly}}^{-1}$  (succinic acid),  $0.06 \pm 0.01 \text{ mol}_{n\text{-POH}} \text{ mol}_{\text{Gly}}^{-1}$  (*n*-propanol) and  $0.05 \pm 0.01 \text{ mol}_{\text{AA}} \text{ mol}_{\text{Gly}}^{-1}$  (acetic acid) (**Fig. 3**). In batches 10 and 11, reducing HTPJ and biotin concentrations resulted in alteration of by-products profile, as the acetic acid and succinic acid yields were decreased by 38 and 40%, respectively. This could be attributed to the lower aspartic- and lactic acid contents in the fermentation medium upon dilution (<http://www.starch.dk/isi/energy/juicefeed.htm> “Last accessed June 2012”; Supplementary S1), which have been reported to be metabolized by some *Propionibacteria* to acetic- and succinic acid (Crow, 1986). In contrast, yields of propionic acid and *n*-propanol were not affected. As a consequence of lowering HTPJ and biotin concentrations, molar ratio of propionic acid to total organic acids was increased from an average of 79 mol% for the initial 9 batches to 84 and 87 mol% for batches 10 and 11, respectively.

### 3.3 Sequential batch fermentation for propionic acid production using increasing glycerol concentrations

In order to determine the maximum convertible glycerol and maximum attainable propionic acid concentrations, five sequential batches with cell recycle were performed using increasing glycerol concentrations (**Fig. 4**). The fermentation was started with an initial cell density of  $8.8 \text{ g}_{\text{CDW}} \text{ L}^{-1}$ . In the initial two batches,  $50 \text{ g L}^{-1}$  glycerol was consumed as described in **Section 3.2**. In the subsequent two batches,  $85 \text{ g L}^{-1}$  glycerol was consumed in 58 and 70 h, yielding  $42.2$  and  $45.3 \text{ g L}^{-1}$  propionic acid, respectively at a rate of  $0.88$  and  $0.77 \text{ g L}^{-1} \text{ h}^{-1}$ .

When propionic acid concentration reached 30 g L<sup>-1</sup>, product inhibition was observed, and glycerol consumption- and propionic acid production rates were correspondingly decreased by 52 and 42% for the former batch, and by 63 and 68% for the latter batch.

The highest concentration of glycerol that was entirely consumed was 120 g L<sup>-1</sup>, which is the highest reported for a batch mode of operation. The initial 90 g L<sup>-1</sup> of glycerol was consumed in 84 h, producing 46.4 g L<sup>-1</sup> propionic acid at a yield of 89 mol%. No substrate inhibition was observed, as propionic acid production occurred at a rate of 1.02 g L<sup>-1</sup> h<sup>-1</sup> for the initial 58 g L<sup>-1</sup> glycerol consumed, and 0.70 g L<sup>-1</sup> h<sup>-1</sup> when considering consumption of initial 81 g L<sup>-1</sup> glycerol. These values are close to the rates observed with 50 and 85 g L<sup>-1</sup> of glycerol in the former runs. This makes glycerol an advantageous substrate compared to glucose and lactose, where substrate inhibition starts at much lower concentrations (Barbirato et al., 1997; Huang et al., 2002). The remaining 30 g L<sup>-1</sup> of glycerol however were consumed at a much lower rate, which resulted in reduction of overall productivity to 0.29 g L<sup>-1</sup> h<sup>-1</sup> (**Fig. 4A**).

Continued fermentation for 200 h showed negligible cell growth, however the cells were still metabolically active and were able to utilize the residual glycerol and produce propionic acid. Nevertheless, the propionic acid concentration was only increased by 4.4 g L<sup>-1</sup>, due to increase in culture volume by 102 mL during this period as a result of base addition. A combined effect of product inhibition, and insufficient nitrogen/vitamin concentration for the high cell density towards the end of the fermentation might explain this effect. The low nitrogen/vitamin source concentration not only affects the cell growth and metabolism, but also lowers the tolerance of *Propionibacteria* cells to propionic acid (Quesada-Chanto et al., 1998). Using concentrated potato juice may improve the fermentation kinetics and allow conversion of higher glycerol concentrations.



### **3.4 Impact of the raw materials on the proposed process**

The use of HTPJ as nitrogen/vitamin source for propionic acid production provides several advantages. The narrow difference between the costs of glycerol (0.2-0.6 USD Kg<sup>-1</sup>) and propionic acid (2-3 USD Kg<sup>-1</sup>) makes the process highly sensitive to the cost of nitrogen/vitamin source. Although yeast extract gives good growth and propionic acid productivity, it is very expensive to be used industrially. Replacement of yeast extract with HTPJ considerably minimizes the cost of nitrogen/vitamin source. Moreover, reducing HTPJ concentration under optimized fermentation conditions has minimal effect on propionic acid productivity and could give a further reduction of HTPJ cost by 25% through shifting between full-strength and half-strength medium after reaching optimum productivity. Using lower HTPJ concentration has an added value as well to the downstream processing through minimizing the concentrations of other organic acids that could interfere with propionic acid purification.

Most industrial and agricultural by-products that have been utilized as N-source were considered to be poor growth media, and required pre-treatment, either chemically or enzymatically, and/or supplementation with other N-additives for enhancing fermentation kinetics (Colomban et al., 1993; Feng et al., 2011). In the process proposed here, the heat-treated potato juice alone was sufficient for the whole process. Also no pre-treatment step was required, which minimizes the number of steps, capitals and investment for large-scale production. Though supplementation with N-additives or pretreatment would improve fermentation kinetics (unpublished data), a techno-economic evaluation for the significance of this step is recommended.

To our knowledge, this is the first report for utilization of agro-industrial by-product as nitrogen/vitamin source in combination with glycerol for propionic acid production. All processes reported earlier utilize either sugar or lactate containing raw materials or a

combination of biodiesel glycerol with expensive nitrogen sources. As demonstrated here, the combination of HTPJ and glycerol in a high-cell-density fermentation provided high fermentation rates, high propionate yield and concentration. Propionic acid represented ~80 mol% of total organic acids; succinic acid and acetic acid were the main acidic by-products and had a ratio of as low as 13.7 and 4.5 g% with regards to propionic acid. When sugar cane molasses was used as a raw material, succinic acid concentration was 10.0 g% of propionic acid while acetic acid was 13.0 g% (Feng et al., 2011). When whey lactose and hydrolyzed corn meal were used, the PA/AA ratio were 3.7 and 4.0  $\text{g}_{\text{PA}} \text{g}_{\text{AA}}^{-1}$ , respectively which is ~60% lower than that with HTPJ and glycerol (Colomban et al., 1993; Huang et al., 2002). Consequently, propionic acid production from potato juice and glycerol is considered more economical if downstream processing cost is taken into consideration.

**Table 1** shows an estimation of raw materials amount and cost required for production of 1 kg propionic acid based on a yield of  $0.7 \text{ g}_{\text{PA}} \text{g}_{\text{Gly}}^{-1}$  and a final propionic acid concentration of  $50 \text{ g L}^{-1}$ . Succinic acid is produced as a major by-product; for each gram of propionic acid produced, approximately 0.1 g of succinic acid could be obtained and could represent a potentially useful co-product. Recently several industrial processes based on fermentation technology are focusing on bio-succinic acid production for eventual use in polymer production (Taylor, 2010). The market price for bio-succinic acid was estimated at 3-5 USD  $\text{Kg}^{-1}$  in 2010 (Taylor, 2010). The revenue from succinic acid would cover the nitrogen/vitamin source cost (**Table 1**), and hence the raw materials cost will be represented only by glycerol. The use of crude glycerol is likely to make the process highly cost-effective provide the fermentation process is not negatively affected. According to earlier reports (Ruhel et al., 2011; Zhang and Yang, 2009), crude glycerol obtained from the biodiesel process, was utilized efficiently and gave higher propionic acid yield compared to pure glycerol. In a preliminary study, we have also observed biodiesel-derived glycerol (glycerine

tech 98%, from Perstorp AB) to be a better substrate for propionic acid production (unpublished data).

The economic viability of the process can be further improved by utilizing the residual cell biomass from the process as starter culture in cheese industry, probiotic or silage preservative (Colomban et al., 1993), or hydrolyzing it for use as nitrogen and vitamin supplement for subsequent fermentations (Feng et al., 2011). Other valuable co-products such as trehalose or vitamin B12 could also be extracted from the cell bleed (Quesada-chanto et al., 1994; Ruhai et al., 2011).

#### **4 Conclusions**

The present study demonstrates the significance of carbon source, nitrogen/vitamin source and process design on production of the platform bulk chemical, propionic acid. It also shows the potential of heat-treated potato juice as an alternative, cheap, renewable nitrogen source. High cell density fermentation allowed faster fermentation rates. Finally, a green economically feasible process was introduced for propionic acid production, which could be highly competitive to the industrially utilized fossil-based process, especially when operated as a biorefinery by integrating production of other valuable products.

#### **5 Acknowledgements**

The Swedish Governmental Agency for Innovation Systems (VINNOVA) is acknowledged for financing the project. Perstorp AB is appreciated for coordination and Lyckeby Starch AB is thanked for supplying potato juice and the required information about potato juice production process. The authors appreciate Dr. Per Persson's contribution to the project.

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## Legends to figures

**Fig. 1** Glycerol consumption and metabolites production using high-cell-density sequential batch fermentation of glycerol by *P. acidipropionici* DSM 4900 with cell recycle for 11 consecutive batches. The figures show [A] concentrations of Glycerol (●) and propionic acid (\*), [B] succinic acid (●) and *n*-propanol (\*), and [C] cell growth represented by Ln(OD). The cultivation medium contained 50 g glycerol, 0.5 mg biotin dissolved in heat-treated potato juice (HTPJ) in a total volume of 1 L. In batches 10 and 11, the concentrations of HTPJ and biotin were 50% lower. Experimental conditions are described in the text.

**Fig. 2** Kinetics of sequential batch propionic acid fermentation using 50 g L<sup>-1</sup> glycerol and HTPJ with *P. acidipropionici* DSM 4900 recycled for 11 sequential batches. The parameters shown are: [A] logarithmic increase in propionic acid volumetric productivity for the sequential batches, [B] changes in propionic acid volumetric production (\*) and glycerol consumption (■) rates (g L<sup>-1</sup> h<sup>-1</sup>) as a function of initial biomass concentration, [C] changes in specific glycerol consumption (■) and propionic acid production (\*) rates, and [D] changes in the ratio of propionic acid concentration (g L<sup>-1</sup>) to biomass (g<sub>CDW</sub> L<sup>-1</sup>) for the different batches.

**Fig. 3** Yields of propionic acid (x), succinic acid (■), acetic acid (▲) and *n*-propanol (◆) from 50 g L<sup>-1</sup> glycerol, and the molar ratio of PA/AA (grey bars) for 11 sequential batch fermentations with cell recycle. Nitrogen and vitamin sources were HTPJ and biotin.

**Fig. 4** Effect of increasing glycerol concentrations on propionic acid fermentation using high-cell-density sequential batch fermentation with *P. acidipropionici* DSM 4900 cells. [A]

Glycerol (●) and propionic acid (\*), [B] succinic acid (●) and *n*-propanol (\*), and [C] Ln(OD). The cultivation medium contained variable amount of glycerol, 0.5 mg biotin dissolved in heat-treated potato juice (HTPJ) in a total volume of 1 L.

## Tables

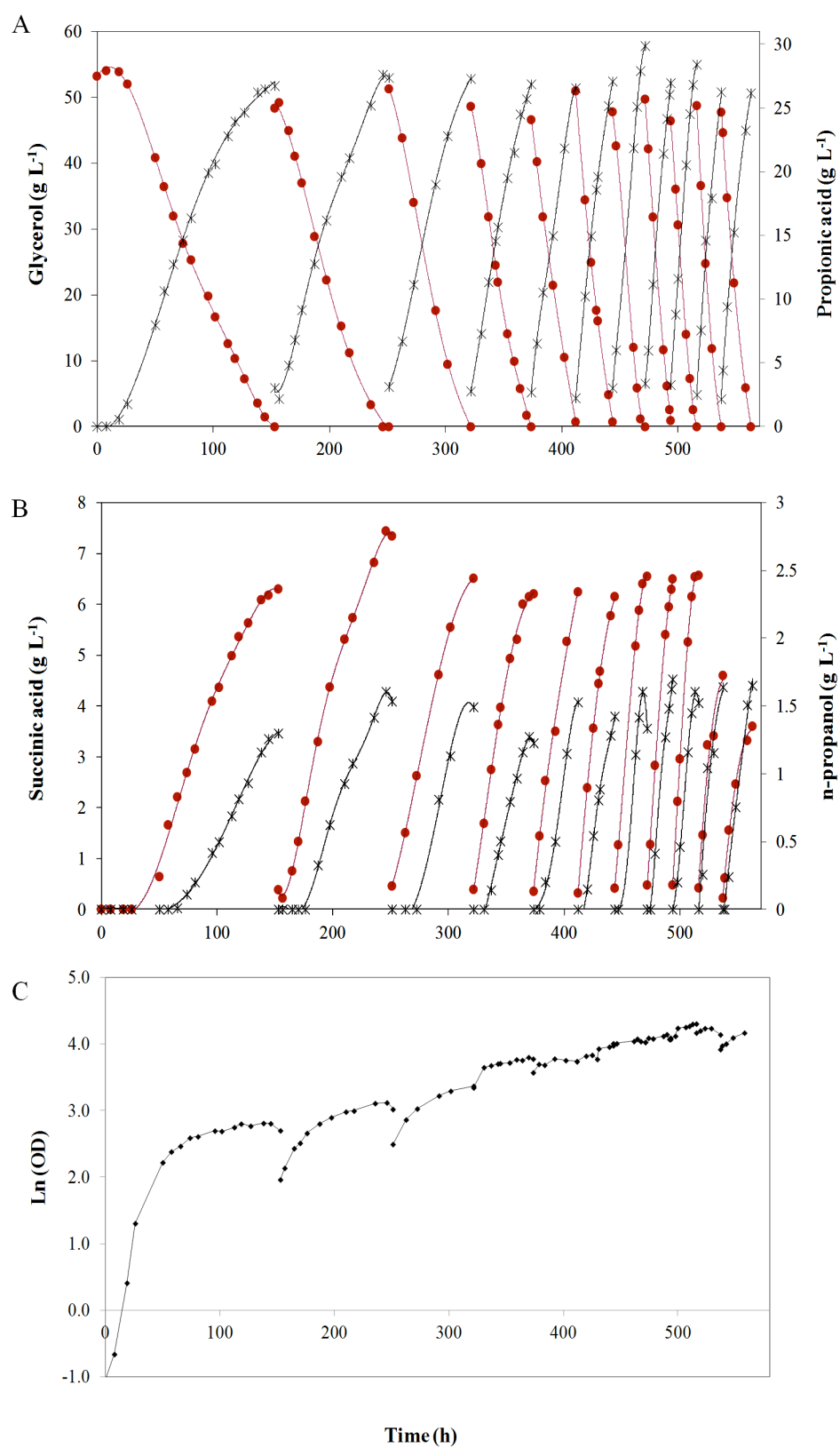
**Table 1** The amount and cost of raw materials per kilogram of propionic acid produced considering yield of  $0.7 \text{ g}_{\text{PA}} \text{ g}_{\text{Gly}}^{-1}$  and  $0.07 \text{ g}_{\text{SA}} \text{ g}_{\text{Gly}}^{-1}$  and propionic acid final concentration of  $50 \text{ g L}^{-1}$

Raw material	Amount	Market price	Cost USD Kg <sup>-1</sup> PA	Reference
Crude glycerol (CG)	$1.43 \text{ g}_{\text{gly}} \text{ g}_{\text{PA}}^{-1}$	200 USD ton <sup>-1</sup>	0.29	Per Persson, 2011 (Personal communication)
Refined glycerol (RG)	$1.43 \text{ g}_{\text{gly}} \text{ g}_{\text{PA}}^{-1}$	600 USD ton <sup>-1</sup>	0.87	Per Persson, 2011 (Personal communication)
Potato juice	1 L/50 g <sub>PA</sub>	15 USD m <sup>-3</sup>	0.3	
Biotin	0.5 mg/50 g <sub>PA</sub>	1800 USD kg <sup>-1</sup>	0.018	Maria Vilorio-Cols, 2011 (Personal communication)
Propionic acid	1 kg	2-3 USD kg <sup>-1</sup>	2 – 3	Shukun Yu, 2011 (Personal communication)
Succinic acid	$0.1 \text{ g}_{\text{SA}} \text{ g}_{\text{PA}}^{-1}$	3-5 USD kg <sup>-1</sup>	0.3 – 0.5	Taylor 2010

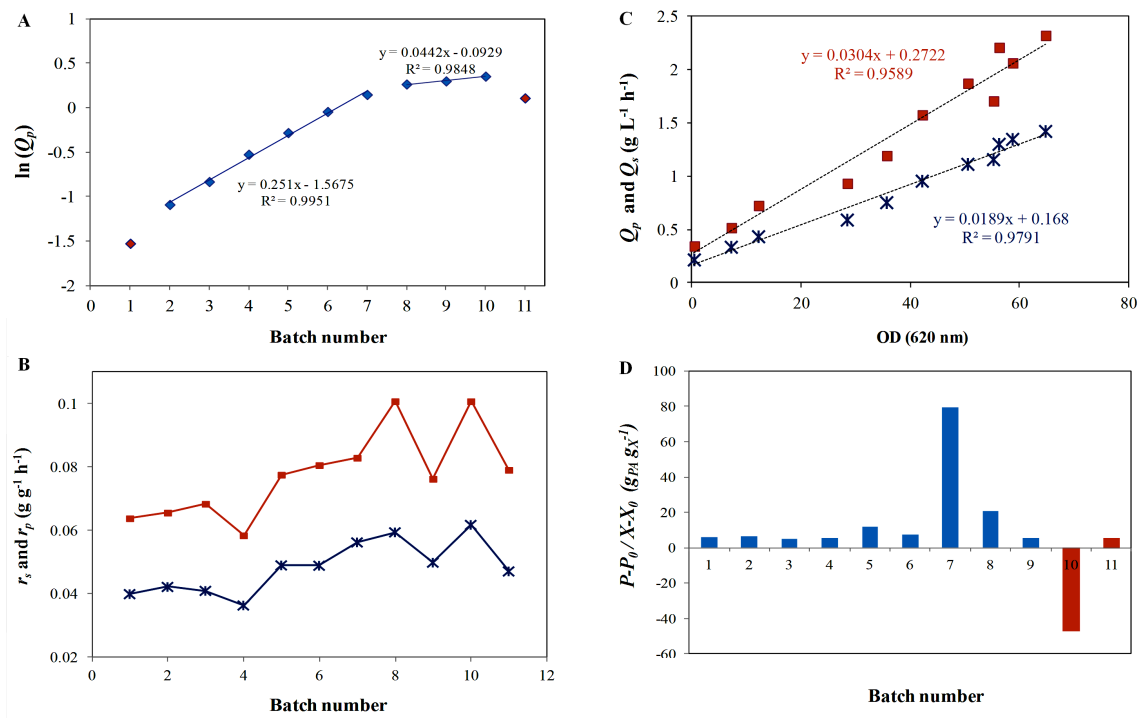


# Figures

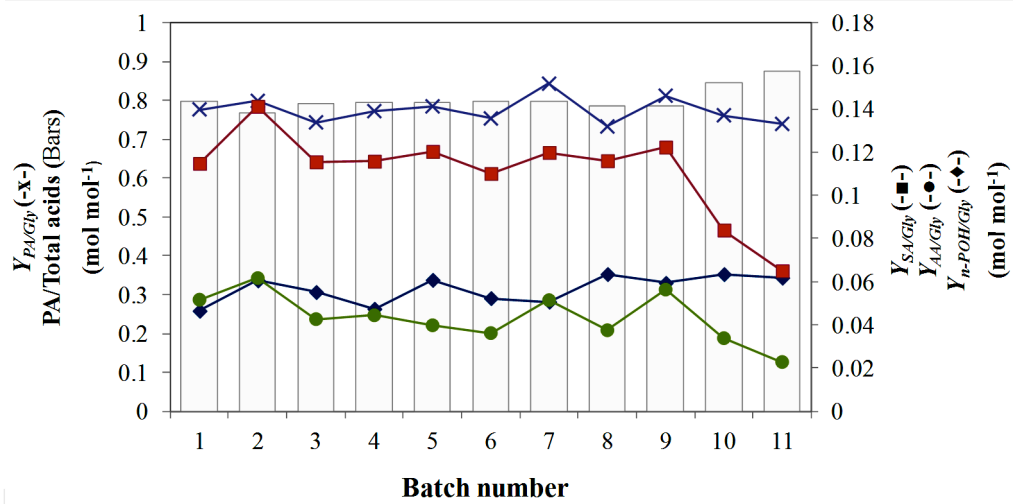
Fig. 1



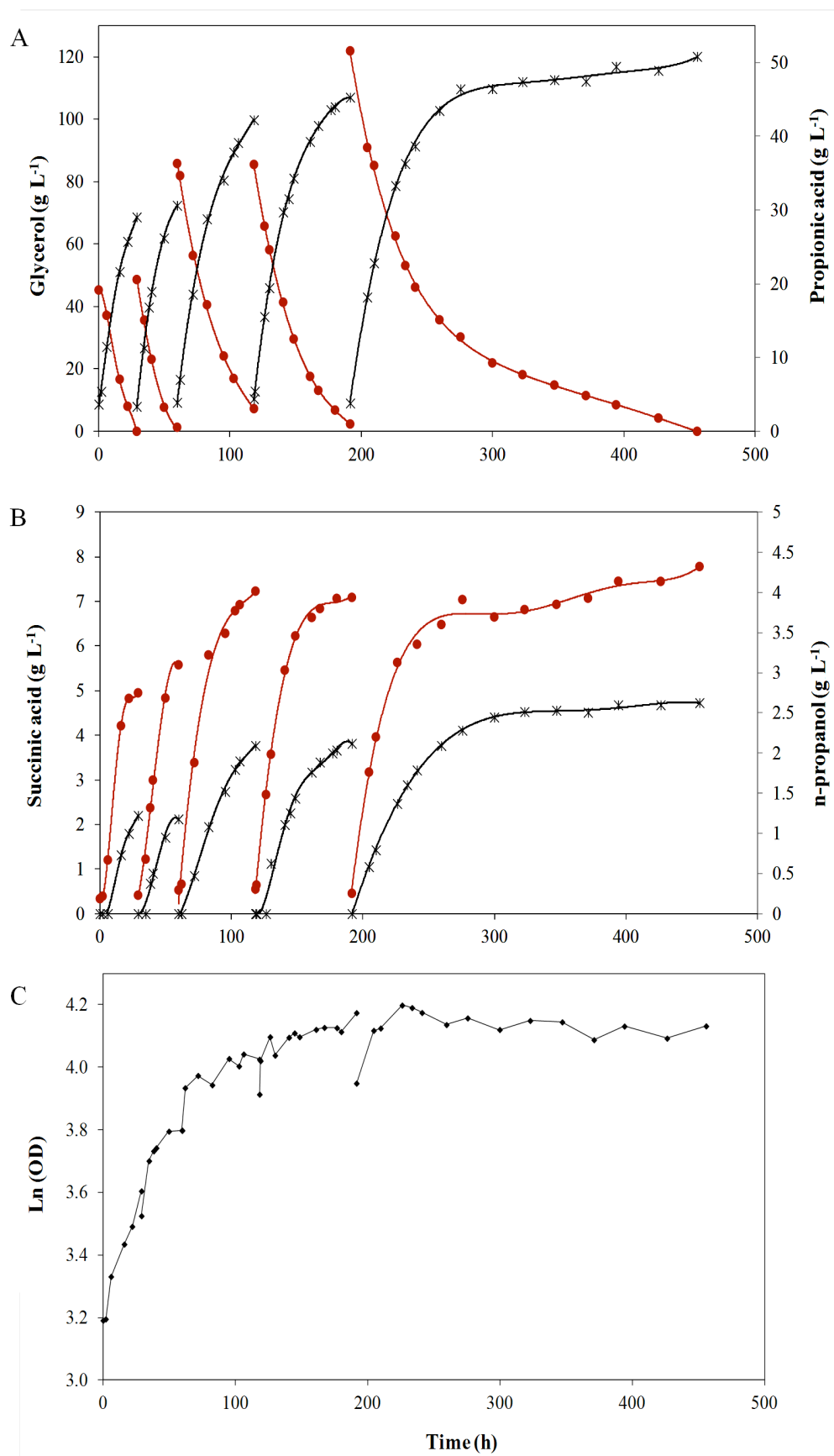
**Fig. 2**



**Fig. 3**



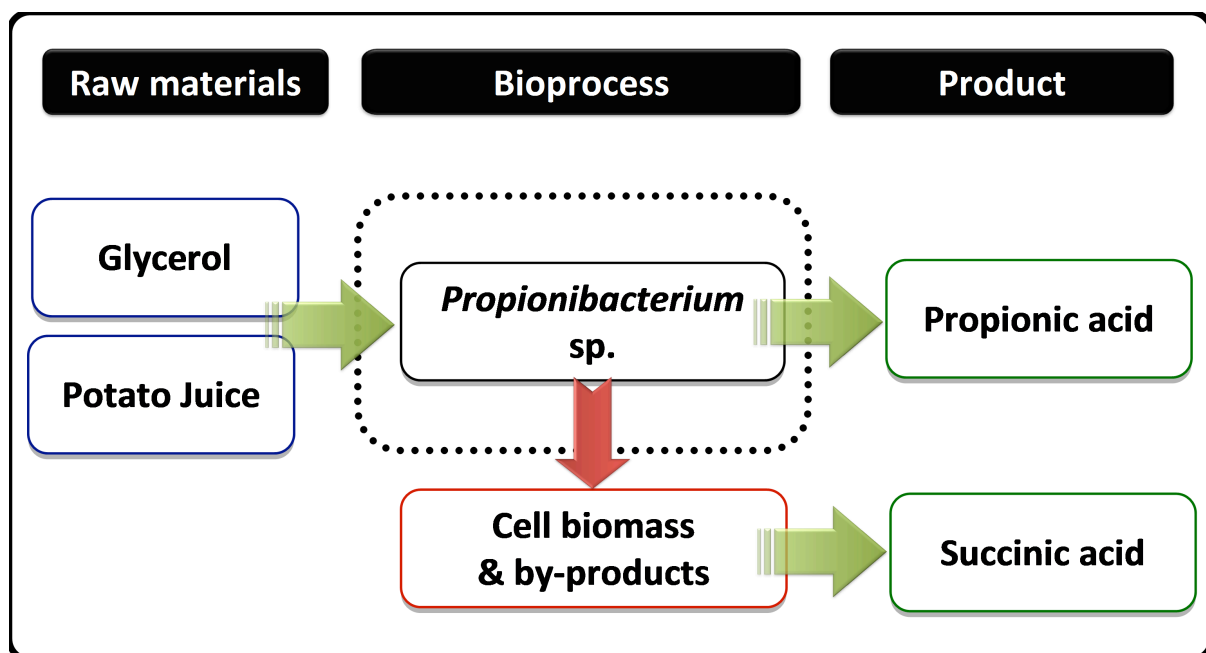
**Fig. 4**



## Research highlights

- Glycerol and potato juice as raw materials for propionic acid production
- Sequential batch fermentation with cell recycle gives high propionate productivity
- Highest glycerol concentration of 120 gL<sup>-1</sup> consumed to give 78 mol% propionic acid
- Cost analysis of raw materials shows economical feasibility of the process

## Graphical abstract



## Electronic supplementary information

# **An economical biorefinery process for propionic acid production from glycerol and potato juice using high cell density fermentation**

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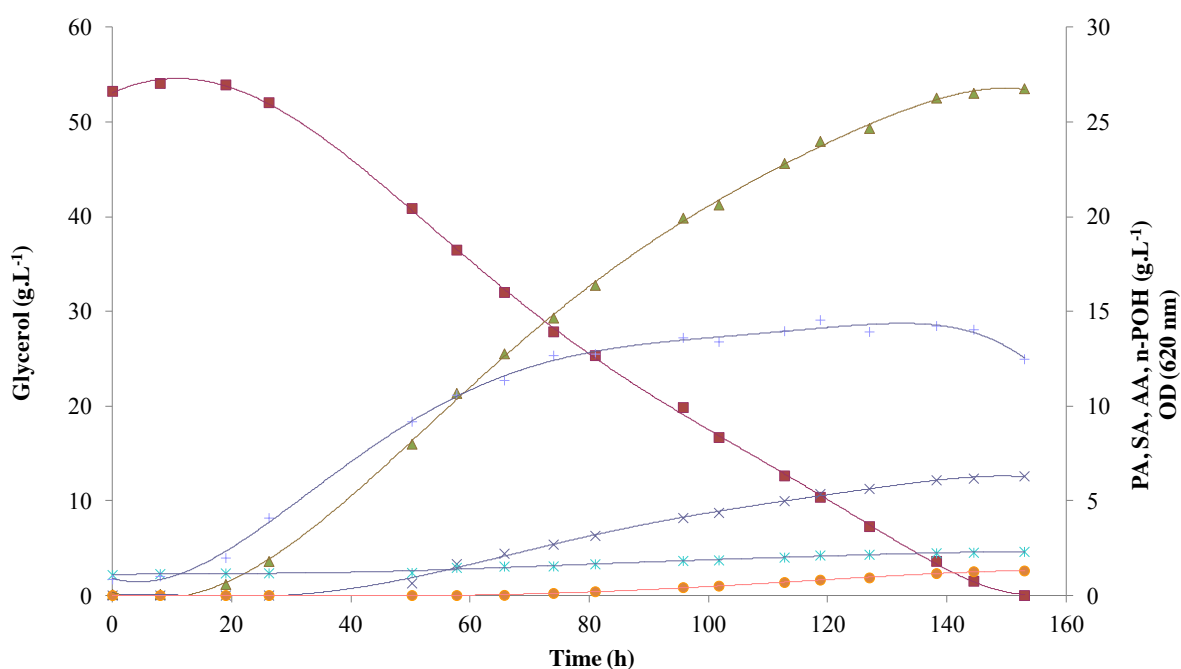
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## Supplementary

### Supplementary S1. Chemical and elemental composition of heat-treated potato fruit juice

Component	Units	Supernatant from separation of heat-treated potato fruit juice
Nitrogen	mg kg <sub>DM</sub> <sup>-1</sup> <sup>(a)</sup>	48000
Potassium	mg kg <sub>DM</sub> <sup>-1</sup>	120000
Phosphorous	mg kg <sub>DM</sub> <sup>-1</sup>	10000
Sulphur	mg kg <sub>DM</sub> <sup>-1</sup>	11600
Calcium	mg kg <sub>DM</sub> <sup>-1</sup>	4350
Magnesium	mg kg <sub>DM</sub> <sup>-1</sup>	7900
Iron	mg kg <sub>DM</sub> <sup>-1</sup>	138
Boron	mg kg <sub>DM</sub> <sup>-1</sup>	23
Manganese	mg kg <sub>DM</sub> <sup>-1</sup>	36
Zinc	mg kg <sub>DM</sub> <sup>-1</sup>	91
Copper	mg kg <sub>DM</sub> <sup>-1</sup>	25
Silicon	mg kg <sub>DM</sub> <sup>-1</sup>	827
Aluminium	mg kg <sub>DM</sub> <sup>-1</sup>	26
Lactic acid	g L <sup>-1</sup>	1.1
Acetic acid	g L <sup>-1</sup>	1.7
<sup>(a)</sup> DM: dry matter		



**Supplementary S2.** Detailed view of a typical batch fermentation for propionic acid production from glycerol using heat-treated potato fruit juice as a nitrogen/vitamin source. The concentration of glycerol (■), propionic acid (▲), succinic acid (x), acetic acid (\*) and n-propanol (●), and the microbial growth as OD at 620 nm (+) are shown.