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**Biotransformation of glycerol to 3-hydroxypropionaldehyde:
improved production by *in situ* complexation with bisulfite in a
fed-batch mode and separation on anion exchanger**

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Abstract

3-Hydroxypropionaldehyde (3HPA) is an important C3 chemical that can be produced from renewable glycerol by resting whole cells of *Lactobacillus reuteri*. However the process efficiency is limited due to substrate inhibition, product-mediated loss of enzyme activity and cell viability, and also formation of by-products. Complex formation of 3HPA with sodium bisulfite and subsequent binding to Amberlite IRA-400 was investigated as a means of *in situ* product recovery and for overcoming inhibition. The adsorption capacity and -isotherm of the resin were evaluated using the Langmuir model. The resin exhibited maximum capacity of 2.92 mmol complex/g when equilibrated with 45 mL solution containing an equilibrium mixture of 2.74 mmol 3HPA-bisulfite complex and 2.01 mmol free 3HPA. The dynamic binding capacity based on the breakthrough curve of 3HPA and its complex on passing a solution with 2.49 mmol complex and 1.65 mmol free 3HPA was 2.01 mmol/g resin. The bound 3HPA was desorbed from the resin using 0.20 M NaCl with a high purity as a mixture of complexed- and free 3HPA at a ratio of 0.77 mol/mol. Fed-batch biotransformation of glycerol (818.85 mmol) with *in situ* 3HPA complexation and separation on the bisulfite-functionalized resin resulted in an improved process with consumption of 481.36 mmol glycerol yielding 325.54 mmol 3HPA at a rate of 17.13 mmol/h and a yield of 68 mol%. Also, the cell activity was maintained for at least 28 h.

Keywords:

3HPA-bisulfite complex; *in situ* recovery; anion exchange resin; adsorption isotherm; column separation; *Lactobacillus reuteri*

1. Introduction

3-Hydroxypropionaldehyde (3HPA) is among the top 10 target chemicals for the biobased industry (Bozell and Petersen, 2010) because of its potential role as a central component in a network of several high volume biorefinery products including 1,3-propanediol (1,3PDO), 3-hydroxypropionic acid (3HP), acrylic acid, malonic acid, acrolein and acrylamide (Bauer et al., 2010; Lüthi-Peng et al., 2002a; Ulmer et al., 2002; Ulmer and Zeng, 2007; Vollenweider and Lacroix, 2004). The need for research and technology development has been identified in order to make 3HPA a marketable product (Bozell and Petersen, 2010). Currently, 3HPA is formed as an intermediate in two chemical processes for production of 1,3PDO from fossil based propylene and ethylene, respectively (Arntz and Wiegand, 1991; Knifton et al., 2003). These processes require unfavorable operational conditions, and do not allow easy isolation of 3HPA (Vollenweider and Lacroix, 2004).

The availability of glycerol as a by-product of biodiesel production has led to an interesting possibility of using it as a renewable non-toxic raw material for production of 3HPA through a dehydration step (Johnson and Taconi, 2007). However, thermal dehydration of glycerol yields the highly toxic acrolein, which upon hydration in presence of sulfuric acid gives 3HPA (Pressman and Lucas, 1942; Redtenbacher, 1843). Several anaerobic microorganisms produce 3HPA by selective dehydration of glycerol in a single reaction step catalyzed by glycerol/diol dehydratase under mild environmental conditions. The aldehyde is an intermediate in metabolic pathways ending in 1,3PDO or a mixture of 1,3PDO with 3HP (Barbirato et al., 1996; Doleyres et al., 2005; Krauter et al., 2012; Sardari et al., 2013). Among the different microorganisms, the probiotic *Lactobacillus reuteri* exhibits significantly higher tolerance towards 3HPA, and converts glycerol to reuterin, a natural antimicrobial that is an equilibrium mixture of 3HPA monomer with its hydrate and dimer (Barbirato et al., 1996; Lüthi-Peng et al., 2002a; Lüthi-Peng et al., 2002b). However, the

major bottleneck in the microbial production of 3HPA is its sensitivity to substrate- and product-mediated inhibition (Gibson et al., 2011; Talarico et al., 1988). Accumulation of 3HPA (above 30.00 mM) results in inhibition of biocatalytic activity of the cells and loss of cell viability through interaction with amino- and thiol- groups in proteins and other molecules in the cells (Chen et al., 2002; Cleusix et al., 2007; Schaefer et al., 2010; Vollenweider et al., 2010).

Different techniques have been investigated for enhancing 3HPA production and protection of the producer microorganism such as engineering of glycerol dehydratase (Gibson et al., 2011), knockout of the genes encoding enzymes catalyzing the reduction of 3HPA to 1,3PDO (Stevens et al., 2011), and *in situ* complex formation with semicarbazide, carbonylhydrazide or sodium bisulfite (Krauter et al., 2012; Rützi et al., 2011; Sardari et al., 2013; Stevens et al., 2013). Complexation of 3HPA with carbonylhydrazide has so far resulted in its production from high glycerol concentration at a quantitative molar yield approaching 1 mol/mol (Krauter et al., 2012). However, liberation of free 3HPA from the complex is problematic requiring harsh conditions (unpublished data). On the other hand, the complex of 3HPA with bisulfite is less strong and can be easily dissociated (Rützi et al., 2011).

Complexation of 3HPA with sodium bisulfite and with bisulfite-functionalized anion exchange resin, respectively, has been reported earlier by Rützi et al. (2011). The bound 3HPA could be eluted from the resin using saturated NaCl solution. However, when applied for *in situ* 3HPA removal, both the bisulfite (at a concentration exceeding 50.00 mM) and the anion exchanger led to severe repression of glycerol bioconversion (Rützi et al., 2011).

We have recently demonstrated that *in situ* complexation with bisulfite during biotransformation of glycerol in a controlled fed-batch process resulted in a considerably prolonged activity of *L. reuteri* cells and increased production of 3HPA (Sardari et al., 2013). The fed-batch mode reduces the inhibition by glycerol, while bisulfite complexation lowers

3HPA interaction with the cells through blocking the highly reactive carbonyl group of the aldehyde. However, the process of 3HPA-bisulfite complex formation follows a thermodynamic equilibrium between the free aldehyde and the complex at a ratio of 1:1, which limits the final concentration and yield of 3HPA (Sardari et al., 2013). Altering this equilibrium by removal of the 3HPA monomer and complex on an anion exchanger could shift the equilibrium and enhance the productivity. Scheme 1 shows the proposed mechanism for *in situ* 3HPA-bisulfite complex formation, binding of the complex to the anion exchanger and desorption using sodium chloride.

In the present study, a process with improved 3HPA productivity was developed by fed-batch transformation of glycerol using resting cells of *L. reuteri* and *in situ* complexation of 3HPA with bisulfite and binding of the free aldehyde and complex to a bisulfite-functionalized anion exchanger. In order to determine the optimal conditions for the system, the conditions for maximal binding of the mixture of 3HPA and its complex with bisulfite to the non-functionalized anion exchanger and their elution from the resin were first investigated.

2. Materials and methods

2.1. Materials

Amberlite IRA-400 (Cl), Amberlite 400 (OH), Amberlite IRN-78 (OH) and Dowex 66, glycerol, 1,3-propanediol, DL-tryptophan, formaldehyde-sodium bisulfite adduct (95%), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), iodine concentrate and starch solution (contains stabilizer, 1wt.% in H₂O) were procured from Sigma-Aldrich (MO, USA). Sodium hydrogen sulfite (39%), hydrochloric acid (37%) and other chemicals were obtained from Merck (Darmstadt, Germany). De Man, Rogosa and Sharpe (MRS) broth (containing per liter: 10 g proteose peptone, 10.0 g beef extract, 5.0 g yeast extract, 20.0 g dextrose, 1.0 g Tween 80,

2.0 g ammonium citrate, 5.0 g sodium acetate, 0.1g magnesium sulphate, 0.05 g manganese sulphate and 2.0 g dipotassium phosphate) was a product of Difco (MD, USA). 3-Hydroxypropionic acid (30% w/v) was provided by Perstorp AB, Sweden.

2.2. Biocatalyst production

L. reuteri DSM 20016 (DSMZ culture collection, Germany) was used for 3HPA production from glycerol. Twenty milliliters of the culture medium containing 55.0 g/L MRS with 20.00 mM glycerol in a 30 mL serum bottle was boiled, bubbled with nitrogen gas, sealed with a rubber cap and then autoclaved at 121 °C for 15 min. This medium was inoculated with 0.2 mL of *L. reuteri* stock culture in 20% v/v glycerol and incubated anaerobically without shaking for 16 h at 37 °C. For the production of cells for batch biotransformation, 5 mL of the culture broth was used to inoculate 500 mL of the same medium in 600 mL bottles and then incubated for 8 h under the conditions mentioned above.

For fed-batch biotransformation, the cells were produced in a 3 L bioreactor (Applikon, Microbial Biobundle, The Netherlands) containing 2 L MRS medium (55.0 g/L) with 20.00 mM glycerol. The medium was inoculated with 20 mL of the freshly prepared inoculum and cultivation continued for 8 h at 37 °C, 200 rpm, and pH 5.5 by addition of 5N NH₄OH. Anaerobic conditions were maintained by continuous bubbling of nitrogen gas.

The cells were harvested by centrifugation of the broth at 15 000 ×g and 4 °C for 10 min in a Sorvall centrifuge (RC5C, USA). The cell pellet was washed once by re-suspension in 50-100 mL of 50 mM sodium acetate buffer pH 5, and re-centrifuged.

2.3. 3HPA preparation and complex formation with sodium bisulfite

Since 3HPA is not available commercially, it was prepared as described elsewhere using the *L. reuteri* cells (Sardari et al., 2013). The cells (7.5 g cell dry weight) were resuspended in

200.00 mM glycerol solution and mixed in a sealed Falcon tube at 37 °C under anaerobic conditions on a rocking table (Mixer 440, Swelab instrument AB, Sweden). After 2 h, the cells were separated by centrifugation at 15 000 ×g and 4 °C for 10 min and the cell free supernatant was used as a source of 3HPA. The solution was mixed with sodium bisulfite (39% w/v) solution (to give a final bisulfite concentration of 80.00 mM) for 30 min at room temperature to give an equilibrium mixture of 3HPA-bisulfite complex and free 3HPA for use in further experiments (Sardari et al., 2013).

2.4. Screening of resins for binding of 3HPA-bisulfite complex and determination of static and dynamic binding capacities

Three strong anion-exchange resins (gel-type) with quaternary ammonium functional groups: Amberlite 400 (Cl), Amberlite 400 (OH) and Amberlite IRN-78 (OH), and one weak macroporous anion-exchange resin with polyamine functional group, Dowex 66 (free base) were used in this study. One gram of each resin was weighed into 50 mL falcon tubes, washed thoroughly and then swelled in 40 mL de-ionized water for 20 h on a rocking table prior to mixing with 10 mL of the solution containing 62.09 ± 1.65 mM 3HPA-bisulfite complex and 45.97 ± 1.93 mM free 3HPA prepared in Section 2.3. The liquid samples (500 μ L) were collected after 0.5, 1, 2, 3 and 4 h of mixing, and finally the resins were allowed to settle down and the liquid was separated. Then the resins were washed twice with 10 mL de-ionized water. Elution of the bound 3HPA-bisulfite complex was investigated by equilibrating with 10 mL of 0.20 M NaCl solution for 2 h with continuous mixing, and intermittent sampling at 0.5, 1 and 2 h.

The static (batch) binding capacity was determined for Amberlite IRA-400 (Cl) (0.1 g dry weight) by equilibrating with different volumes of freshly prepared 3HPA-bisulfite complex solutions containing different amounts of the complex as follows: 15 mL (0.04 ± 0.00 , $0.11 \pm$

0.00, 0.22 ± 0.01 , 0.43 ± 0.02 and 0.93 ± 0.04 mmol complex, respectively), 30 mL (0.15 ± 0.01 , 0.29 ± 0.01 , 0.59 ± 0.02 and 1.88 ± 0.07 mmol complex, respectively), and 45 mL (0.22 ± 0.01 , 0.45 ± 0.03 , 0.90 ± 0.05 and 2.74 ± 0.02 mmol complex, respectively) for 2 h. Samples were collected and analyzed for the concentration of unbound complex at equilibrium, and the binding capacity (amount of the complex bound per gram resin) was calculated for each solution and -concentration. The experiment was performed in two independent replicates and the presented data are the mean of the two replicates \pm SD.

The dynamic binding capacity and breakthrough curve were determined using a chromatographic column (0.5 cm internal diameter \times 20 cm height) packed with 1.0 g Amberlite IRA-400 (Cl) resin pre-swollen as described earlier in this section. 3HPA-bisulfite complex solution (38.4 mL) containing 64.95 ± 1.75 mM complex and 43.05 ± 1.75 mM free 3HPA, was passed over the column at a flow rate of 0.16 mL/min using a peristaltic pump (Alitea, Sweden). The effluent was collected in fractions of 1.60 mL using a fraction collector (LKB Bromma, 2212 Helirac, Sweden) and the concentration of the complex and free aldehyde in each fraction was measured. The experiment was performed in two independent replicates and the presented data are the mean of the two replicates \pm SD.

2.5. Elution and separation of 3HPA

For determination of optimum conditions for elution of 3HPA, 1.0 g Amberlite IRA-400 (Cl) equilibrated with 5 mL product mixture containing 74.13 ± 0.45 mM 3HPA-bisulfite complex and 59.61 ± 1.23 mM free 3HPA in a 50 mL tube for 2 h, and washed twice with 10 mL deionized water, was treated with 10 mL of 0.20, 1.00 and 3.00 M sodium chloride, respectively. Samples were taken after 0.5, 1 and 2 h, and analyzed to measure the concentrations of eluted complex and free 3HPA.

Five milliliter product solution (containing 61.08 ± 7.18 mM 3HPA-bisulfite complex and 49.41 ± 3.66 mM free 3HPA) was first loaded on the chromatographic column (0.5×20 cm) packed with pre-swollen 1.0 g Amberlite IRA-400 (Cl) resin in de-ionized water. The resin was then washed with 20 mL deionized water, and the bound 3HPA-bisulfite complex and free 3HPA was eluted using 20 mL of 0.20 M sodium chloride. All the steps were done at a flow rate of 0.16 mL/min. The eluted fraction was then analyzed for free- and complexed-aldehyde. The recovered 3HPA was analyzed for purity. The experiment was performed in two independent replicates and the presented data are the mean of the two replicates \pm SD.

2.6. Fed-batch biotransformation of glycerol with in situ 3HPA removal

Fed-batch biotransformation of glycerol using *L. reuteri* cells was done using the setup shown in Fig. 1. The 3 L Applikon bioreactor was coupled to a tangential flow microfiltration module (Pellicon XL, $0.45 \mu\text{m}$, 50 cm^2 filtration area, Millipore, Bedford, MA) and a column (0.5×53 cm) packed with 50.0 g (dry weight) of bisulfite functionalized Amberlite IRA-400. The latter was prepared by swelling the resin initially in 500 mL deionized water in 500 mL flask for 12 h, packing in the column, and then passing the sodium bisulfite solution (39%) upwards at a rate of 6 mL/min. When the pH of the effluent equals that of the sodium bisulfite solution (approximately 10 min), 150 mL sodium bisulfite solution was recirculated over the column overnight. The column was then washed by passing deionized water upwards at a rate of 2 mL/min for 15 h.

L. reuteri cells (5.4 g dry weight) (Section 2.2) were re-suspended in 1 L solution containing 21.70 mmol glycerol and 10.86 mmol sodium bisulfite in the 3 L bioreactor, and biotransformation was performed at $37 \text{ }^\circ\text{C}$, 200 rpm and pH 5 maintained by addition of 5N NH_4OH . After 1 h of batch biotransformation, 993.6 mL of feeding solution containing 802.00 ± 3.80 mM (73.9 g/L) glycerol and 401.01 ± 3.80 mM (41.7 g/L) sodium bisulfite

was fed at a rate of 0.92 mL/min. The reaction mixture was recycled through the microfiltration unit at a rate of 30-40 mL/min, and the cell free permeate was pumped upwards through the column packed with bisulfite-functionalized Amberlite IRA-400 at a rate of 6 mL/min and back to the bioreactor. Samples were collected frequently from the reactor and analyzed for the concentrations of glycerol, 1,3PDO, 3HP, free 3HPA, total bisulfite and free-bisulfite. The concentration of total 3HPA complex in solution and bound to the resin was determined according to Eqn. 1. The concentration of 3HPA-bisulfite complex in solution (not bound to the resin) was determined using Eqn. 2, while the concentration of total 3HPA in solution (not bound to the resin) was determined using Eqn 3.

Total 3HPA complex in solution and bound to resin (mM)

$$= [\text{Consumed glycerol} - (1,3\text{PDO} + 3\text{HP} + \text{free 3HPA})] \quad (\text{Eqn 1})$$

$$3\text{HPA complex in solution (mM)} = [\text{Total bisulfite} - \text{free bisulfite}] \quad (\text{Eqn 2})$$

$$\text{Total 3HPA in solution (mM)} = [\text{Free 3HPA} + (\text{Total bisulfite} - \text{free bisulfite})] \quad (\text{Eqn 3})$$

The experiment was performed in two independent replicates and the presented data are the mean of the two replicates \pm SD.

2.7. Quantitative analyses

The concentration of 3HPA was determined by the modified colorimetric method using acrolein as standard (Circle et al., 1945), in which 0.75 mL DL-tryptophan (10 mM solution in 50 mM HCl) and 3 mL concentrated HCl were added to 1 mL of a properly diluted sample. The mixture was incubated at 37 °C for 20 min and the absorbance of the resulting purple color was read at 560 nm using an UV/Vis spectrophotometer (Ultrospec 1000, Pharmacia biotech, Sweden).

For determination of total hydrogen sulfite, the sample (990 μ L) diluted in 0.20 M potassium phosphate buffer (pH 7.0) was mixed with 10 μ L 5,5'-dithiobis(2-nitrobenzoic

acid) (DNTB) (4% w/v in dimethyl sulfoxide) at 25 °C for 10 min yielding a yellow colored product (5-mercapto-2-nitrobenzoate) (MNB) which was measured at 412 nm (Rütti et al., 2011). The free bisulfite ion concentration was determined by titration with iodine concentrate (12.7 g I₂ + 20 g KI) that was added to 10 µL sample, 3 mL water, and 500 µL starch solution (1% w/v) until a stable violet color of starch-iodine complex was developed (Rütti et al., 2011).

The purity of 3HPA was determined using gas chromatography (GC, Varian 430-GC, Varian, USA) equipped with Factor Four Capillary column, VF-1 ms (Varian, 15 m × 0.25 mm × 0.25 µm film thickness) and a flame ionization detector. The initial column oven temperature was increased from 50 °C to 250 °C at a rate of 20 °C/min. The samples, diluted with acetonitrile to a concentration of 0.1-1.0 mg/mL, were injected in a split injection mode of 10% at 275 °C. The temperature for detection was 275 °C.

Quantification of glycerol, 1,3PDO and 3HP was done by HPLC (JASCO) using an Aminex HPX-87H chromatography column connected to a guard column (Biorad, Richmond, CA, USA) maintained at 65 °C using a column oven (Shimadzu). Samples from the bioreactor were diluted with Milli-Q quality water and mixed with 20% v/v sulfuric acid (20 µL/mL sample) and then filtered. Forty microliter of the sample was injected in a mobile phase of 0.50 mM H₂SO₄ flowing at a rate of 0.4 mL/min. All the compounds were detected using RI-detector (ERC inc., Kawaguchi, Japan), and 3HP concentration was confirmed by detection at 215 nm using a UV detector (Jasco).

3HPA-bisulfite adduct was analyzed in Sections 2.3, 2.4 and 2.5 using a modification of a method reported by Yin et al. (2010) as described in Sardari et al. (2013), involving reaction with o-phthalaldehyde (OPA) and ammonium in alkaline medium yielding a highly fluorescent product. The analysis was done using a Jasco HPLC (Tokyo, Japan) equipped with two pumps for pumping mobile phase A (prepared by dissolving 0.67 g OPA in 3.75 %

v/v aqueous ethanol and diluting to 1 L using Milli-Q quality water) and mobile phase B (composed of 6.75 g borax and 0.133 g NH₄Cl in 1 L Milli-Q water with pH adjusted to 10.7 using NaOH). The two mobile phases were pumped isocratically at a flow rate of 0.5 mL/min divided equally between the two pumps, and mixed in a mixing chamber just prior to the injector. Five microliter of the properly diluted sample was injected into the mobile phase stream, passed through a mixing loop (273 cm length) placed in a Shimadzu column oven (Tokyo, Japan) at 65 °C. The eluted compounds were detected using fluorescence detector (Jasco-FP-920) set at excitation and emission wavelengths of 362 nm and 423 nm, respectively. Formaldehyde-sodium bisulfite adduct (95%) was used as standard.

3. Results and discussion

3.1. Screening of resins for binding 3HPA-bisulfite complex

Screening of different anion exchange resins for binding the 3HPA-bisulfite complex (0.62 mmol) showed Amberlite IRN-78 to have the highest binding capacity -adsorbing the entire complex amount of 0.62 mmol/g, while Amberlite IRA-400 (OH), Amberlite IRA-400 (Cl) and Dowex 66 showed binding capacities of 0.43, 0.33 and 0.25 mmol complex/g resin, respectively. In general, anion exchange resins with OH⁻ counter ions exhibited higher binding for the complex due to the lower electronegativity of OH⁻ ions, which was easily replaced by bisulfite ions. However, elution of the bound complex from these resins was not quantitative. A similar observation has been reported earlier (Rütti et al., 2011). The recovered complex was as low as 0.04 mmol (6.5 % of the amount bound) from Amberlite IRN-78, and reached 0.12 mmol (27%) and 0.15 mmol (60%) from Amberlite IRA-400 (OH) and Dowex 66, respectively. The highest recovery was 0.23 mmol (69%) from Amberlite IRA-400 (Cl), which was hence chosen for further investigations.

3.2. Determination of maximum binding capacity and adsorption isotherm

The binding capacity of 1.0 g Amberlite IRA-400 (Cl) for the 3HPA-bisulfite complex was investigated with different volumes (15-60 ml) of the solution containing the complex in the concentration range of 3.00-65.00 mM (Fig. 2). The binding capacity of the resin was found to be dependent on the volume of the solution used for equilibration and resulted in three adsorption curves (Fig. 2A). The adsorption curve using 60 ml volume (not shown) was very close to that of 45 ml suggesting similar adsorption behavior and that a maximum binding capacity was attained.

The adsorption curves were analysed by Langmuir adsorption isotherm applied at adsorption equilibrium (Eqn 4),

$$C_e/q_e = 1/(q_{max} \cdot k_l) + (C_e/q_{max}) \quad (\text{Eqn 4})$$

where C_e is the equilibrium concentration (mM) of 3HPA-bisulfite complex, q_e and q_{max} are the equilibrium and maximum adsorption capacity of 3HPA-bisulfite complex (mmol/g resin), respectively, and k_l is the Langmuir adsorption equilibrium constant (L/mmol) (Hajizadeh et al., 2010). A plot of C_e/q_e versus C_e showed a linear relation and was used for calculation of the Langmuir constants (Fig 2B, Table 1). The capacity of the Amberlite IRA-400 (Cl) for single charged anions has earlier been reported to be 2.18 mmol/g dry resin based on conversion from the volumetric capacity of 1.4 meq/mL provided by the supplier (Rütti et al., 2011). For the three different volumes (15, 30 and 45 mL) of 3HPA-bisulfite complex solution, the maximum capacities, q_{max} of 1.00 mmol/g resin ($C_e/q_e = 1.00 C_e + 6.80$), 1.71 mmol/g resin ($C_e/q_e = 0.58 C_e + 1.33$) and 2.92 mmol/g resin ($C_e/q_e = 0.34 C_e + 1.24$), were obtained (Table 1).

3.3. Binding of 3HPA-bisulfite complex to Amberlite IRA-400

Fig. 3 shows the breakthrough curve of 3HPA and its complex with bisulfite on passing 38.4 mL of solution with 1.65 ± 0.07 mmol free 3HPA and 2.49 ± 0.07 mmol complex over a column of 1.0 g Amberlite IRA-400 (Cl). Complete binding of the free and complexed aldehyde was observed for the initial 6.4 ml solution volume followed by rapid increase in their concentrations in the effluent until 25.6 mL, and then reaching a plateau around 32 mL. The concentration of total 3HPA in the effluent corresponded to 86% of that in the initial load and the ratio of the complex to free aldehyde (mol/mol) in the last 14.4 mL of the effluent was constant around 1.7:1. The maximum binding capacity of the resin calculated by material balance of the total 3HPA in the load and the effluent was 2.01 ± 0.22 mmol/g, which is close to the theoretical binding capacity of the resin (2.18 mmol/g) and represent 69% of the actual static binding capacity of the resin (2.92 mmol/g) determined in Section 3.2.

The breakthrough curve in Fig. 3 has a typical S-shape with a steep slope extending over 20 mL. In contrast, the breakthrough curve obtained by Rützi et al. (2011) by adsorption of 3HPA to the bisulfite-functionalized resin was very shallow, extending over ~90 mL with the 3HPA concentration in the effluent being ~75% of the initial concentration. The difference in the two cases may be explained by the differences in the interaction: the pre-formed 3HPA-bisulfite complex can interact directly with the positive charge on the Amberlite IRA-400 resin, whereas when free 3HPA solution (an equilibrium mixture of monomer, dimer and hydrate) is loaded only the monomer will interact with the bisulfite-functionalized resin. According to the concentration dependent distribution of the components of 3HPA mixture, the hydrate, dimer and other derivatives are most dominant up to 3HPA concentration of 5.00 M, which results in a shallow breakthrough curve. Hence, adsorption of free 3HPA is preferred in a batch mode or in a continuous mode at low flow rate.

The interaction of 3HPA with Amberlite IRA-400 (Cl) could occur via three mechanisms: a) direct exchange of the counter ions (Cl) with the complex, b) exchange of Cl⁻

ions with free bisulfite ions present in the solution and subsequent binding of free 3HPA to the bisulfite-functionalized groups, and c) direct binding of free 3HPA to Amberlite IRA-400 (Cl). Once in the effluent, the free and complexed 3HPA undergo redistribution to adjust the ratio to an optimum of ~ 1.1 mol complex/mol free aldehyde.

3.5. Elution of 3HPA-bisulfite complex from Amberlite IRA-400

Elution of 3HPA from bisulfite functionalized Amberlite IRA-400 using saturated NaCl solution has been shown earlier (Rütti et al. 2011). We investigated the elution of 3HPA and its complex from the resin using 0.20-3.00 M NaCl. There was only a slight variation in the amount of the aldehyde eluted: 0.35 mmol (0.12 mmol complex + 0.24 mmol free), 0.41 mmol (0.14 mmol complex + 0.26 mmol free), and 0.42 mmol (0.16 mmol complex + 0.27 mmol free) with 0.20, 1.00 and 3.00 M NaCl, respectively, corresponding to 84%, 98.2%, and 97.8% recovery of bound 3HPA. The ratio of the complex to free aldehyde in the eluted fraction was 0.48, 0.55 and 0.58 mol/mol for 0.20 M, 1.00 M and 3.00 M NaCl, respectively, compared to a ratio of 1.24 mol complex/mol free 3HPA for the loading solution. This indicates that NaCl was able to break the complex partially and liberate free 3HPA. Since the eluted amount of 3HPA with the different salt concentrations was not very different, the lowest salt concentration of 0.20 M was preferred for subsequent experiments.

Fig. 4 shows the chromatographic profile of binding the aldehyde from a solution of 0.29 ± 0.02 mmol complex and 0.25 ± 0.02 mmol free 3HPA, glycerol and 1,3PDO to 1.0 g resin in a column, followed by washing with deionized water and elution with 0.20 M NaCl. Quantitative recovery of 3HPA was achieved with a high purity as a mixture of complexed- and free 3HPA (Table 2). As seen in Fig. 5, the 3HPA was totally free from glycerol and 1,3PDO present initially in the reaction mixture. Even the ratio of complex to free 3HPA (mol/mol) was decreased from 1.16 in the load to 0.77 in the effluent. For removal of the

residual bisulfite and sodium chloride, freeze drying of the mixture followed by extraction of 3HPA using ethanol may be applied (Rütti et al., 2011). On the other hand, the thermodynamic equilibrium within the mixture of free 3HPA with 3HPA-bisulfite complex allowed its direct bioconversion to 3HP without further purification (manuscript in preparation).

3.6. In situ 3HPA complexation and separation during bioconversion

In our earlier study, we have demonstrated that a fed-batch system with *in situ* 3HPA-bisulfite complex formation reduced the inhibitory effects of glycerol, 3HPA and bisulfite on the glycerol conversion to 3HPA (Sardari et al., 2013). The effect of modifying the system further by incorporating the removal of 3HPA and its complex by adsorption on Amberlite IRA-400 on the bioconversion efficiency and operational stability of the cells was now evaluated. The inhibitory effect of the resin against the cells was avoided through keeping it in an external loop over which only the cell-free permeate was circulated and then sent back to the reactor. This prevented the interaction of the ion exchange resin with the negative charge on the cell surface. Using the native Amberlite IRA-400 however resulted in a low yield of 3HPA that was attributed to the release of chloride counterions into the reaction mixture, which is inhibitory for biocatalyst activity (data not shown; Lüthi-Peng et al., 2002b). Hence, the resin was pre-functionalized with bisulfite prior to use that would bind 3HPA as well as the complex (to the unmodified sites). The amount of sodium bisulfite for *in situ* complexation was calculated based on an equimolar conversion of glycerol to 3HPA. Since a bisulfite-functionalized resin was used, the molar ratio of glycerol to sodium bisulfite in the feeding solution was adjusted to 2 mol/mol, which is lower than the optimum ratio for complexation of 1.30 mol 3HPA/mol sodium bisulfite determined earlier (Sardari et al., 2013). This will maintain a relatively low concentration of free bisulfite in the bioconversion

solution. The biotransformation process was started with 21.70 mM glycerol and 10.86 mM sodium bisulfite in a batch mode for 1 h after which feeding of glycerol:bisulfite (802.00 : 401.01 mM) mixture was started at a rate of 0.92 mL/min (44.27 mmol glycerol/h) with simultaneous re-circulation of the cell-free permeate through the Amberlite IRA-400 (SO₃H) column. The pH chosen was 5 since it is the optimum for bioconversion of glycerol to 3HPA (Lüthi-Peng et al., 2002b), as well as for 3HPA-bisulfite complex formation (Sardari et al., 2013).

During the initial 5 h of biotransformation, almost complete conversion of glycerol was achieved (Fig. 6A). Subsequently, the concentration of glycerol in the effluent increased gradually and reached 167.79 ± 22.27 mM (15.5 g/L) at the end of the biotransformation process. Overall 481.36 ± 41.91 mmol (44.3 g) glycerol was consumed at an overall rate of 25.34 mmol/h (2.3 g/h). Glycerol accumulation could be reduced by using cells with higher glycerol dehydratase activity (van Pijkeren et al., 2012). The concentration of free 3HPA during the first hour was higher than that of the 3HPA complex due to the slow kinetics of complexation, and then stabilized around 66.68 ± 3.99 mM after 7 h and till the end of biotransformation (Fig. 6A). The total 3HPA amount reached 325.54 ± 8.74 mmol (24.1 g) formed at a rate of 17.13 mmol/h (1.3 g/h) and a molar yield of 68 mol% of glycerol. Equimolar amount of 1,3PDO and 3HP (77.89 \pm 16.58 mmol each) were also produced and represented the main by-products formed. The overall molar ratio of total 3HPA to by-products (1,3PDO and 3HP) was 2.09:1. Both 1,3PDO and 3HP are important platform chemicals and building blocks for other chemicals and polymers in a bio-based industry, and may thus constitute valuable co-products (Dishisha, 2013).

The average ratio of 3HPA-bisulfite complex (in solution and bound to resin) to free 3HPA was found to be 1.43 ± 0.17 mol/mol for the last 10 h. The profile of the 3HPA formation showed an initial production rate of 26.09 mmol/h (1.9 g/h) between 2 and 9 h of

biotransformation (Fig. 6B), after which the rate was reduced to 9.92 mmol/h (0.7 g/h) till the end of the experiment, probably due to the saturation of the resin. The amount of 3HPA bound to the resin reached 94.21 ± 3.15 mmol (1.88 mmol/g resin) after 9 h and a maximum of 113.17 ± 9.39 mmol after 11 h (2.26 mmol/g resin) and remained unchanged until the end of the 19 h. Substitution of the saturated resin with a fresh one at this stage might restore the production rate. Another possible explanation for this inhibition is the concentration of free bisulfite in the solution which exceeded 50.00 mM at 9 h, which was reported to be inhibitory for glycerol biotransformation (Rütti et al., 2011).

After 19 h of biotransformation, the feed was stopped and the reaction was allowed to proceed further for additional 9 h. During this period, partial consumption of the residual glycerol was achieved and the amount of 3HPA in the solution reached 388.77 mmol (28.8 g).

Hence, fed-batch biotransformation with *in situ* complex formation and separation of the complex considerably enhanced the 3HPA production rate and the final 3HPA amount almost 1.7 fold in 19 h as compared to that in the process with only *in situ* complexation with bisulfite (Sardari et al., 2013). Moreover, the biocatalyst activity was maintained for at least 28 h. The amount of 3HPA produced per gram biocatalyst was increased from 2.74 g/g with *in situ* complex formation (Saradari et al., 2013) to 5.33 g/g with *in situ* complexation and removal. The inhibition exerted by the resin on the biocatalyst activity was alleviated through introduction of cell recycling system. Similar systems have been used at industrial and pilot scales for production of various compounds including ethanol and lactic acid (Chematur Engineering Group; Venus, 2012), which shows the scalability of the proposed process.

4. Conclusion

In contrast to the earlier report (Rützi et al. 2011), this work clearly demonstrates the potential of 3HPA complexation with bisulfite and subsequent binding to an anion exchange resin for *in situ* recovery of 3HPA with significantly improved productivity and operational lifetime of the microbial biocatalyst and even decreased by-product content. Although the capacity of complexation with bisulfite is lower than that with carbonylhydrazide, the dissociation of 3HPA-bisulfite complex is easier. The system will be investigated further with an overproducing strain of 3HPA and using industrial grade glycerol.

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Table 1. Langmuir constants of adsorption of 3HPA-bisulfite complex on Amberlite IRA-400 (Cl). The data represent the mean of two independent replicates \pm SD.

3HPA-bisulfite complex (mL)	C_e (mM)	q_e (mmol/g dry resin)	q_{max} (mmol/g dry resin)	k_l (L/mmol)	R^2
15	58.31 ± 0.00	0.94 ± 0.00	1.00	0.15	0.97
	25.56 ± 0.30	0.67 ± 0.05			
	11.38 ± 0.00	0.54 ± 0.00			
	4.44 ± 0.00	0.45 ± 0.00			
	1.05 ± 0.02	0.29 ± 0.00			
30	57.28 ± 3.44	1.69 ± 0.32	1.71	0.44	1.00
	14.78 ± 0.31	1.41 ± 0.09			
	5.86 ± 0.74	1.18 ± 0.13			
	1.58 ± 0.85	0.99 ± 0.21			
45	54.66 ± 1.36	2.79 ± 0.37	2.92	0.28	1.00
	15.08 ± 1.73	2.17 ± 0.24			
	5.81 ± 1.54	1.86 ± 0.42			
	1.89 ± 0.19	1.39 ± 0.05			

Table 2. Binding and elution of 3HPA-bisulfite complex and free 3HPA on 1 g Amberlite IRA-400 (Cl) in a column. The data represent the mean of two independent replicates \pm SD.

Step	3HPA amount (mmol)			Ratio ^(a)	Purity (%)	Recovery (%)
	Complex	Free	Total			
Loaded	0.31 ± 0.04	0.25 ± 0.02	0.55 ± 0.08	1.24	43.5	ND
Washing (0 M NaCl)	0.01 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.50	ND	5.4
Elution (0.20 M NaCl)	0.24 ± 0.02	0.31 ± 0.01	0.55 ± 0.03	0.77	> 99 ^(b)	100

^(a) mol complex/mol free 3HPA
^(b) as mixture of complex and free 3HPA

Figure captions

Fig. 1. Bioreactor system used for biotransformation of glycerol to 3HPA using resting cells of *L. reuteri*, with *in situ* complexation of 3HPA and recovery on an anion exchange resin. The cells were retained by a tangential flow microfiltration module (3) integrated with the bioreactor (1). Pump (2) was used to feed the mixture of glycerol and sodium bisulfite to the bioreactor at a rate of 0.92 mL/min. The reaction suspension with the cells was circulated between the reactor and the microfiltration unit at a rate of 30-40 mL/min using pump (4). The cell free permeate was circulated to the chromatographic column packed with 50.0 g Amberlite IRA-400 (Cl) resin pre-functionalized with sodium bisulfite (6) using pump (5) at a rate of 6 mL/min.

Fig. 2. Adsorption equilibrium between the amount of bound complex to Amberlite IRA-400 (Cl) and the concentration of remaining complex in solution (equilibrium concentration). (A) Plot of the bound complex amount versus the equilibrium concentration using different volumes of 3HPA-bisulfite solution: (◆) 15 mL, (■) 30 mL and (●) 45 mL. (B) Adsorption isotherm of 3HPA-bisulfite complex at different initial volumes of loading solution: (◆) 15 mL ($C_e/q_e = 1.00 C_e + 6.80$), (■) 30 mL ($C_e/q_e = 0.58 C_e + 1.33$), and (●) 45 mL ($C_e/q_e = 0.34 C_e + 1.24$). The curves represent Langmuir adsorption isotherm model applied to the data. C_e is the equilibrium concentration of 3HPA-bisulfite complex in mM, and q_e is the equilibrium adsorption capacity of 3HPA-bisulfite complex in mmol/g resin. The data represent the mean of two independent replicates \pm SD.

Fig. 3. Breakthrough curve of free 3HPA, 3HPA-bisulfite complex, and total 3HPA on loading a solution (38.4 mL) containing 108.00 ± 0.00 mM total 3HPA (▲) [64.95 ± 1.75 mM 3HPA-bisulfite complex (◆) and 43.05 ± 1.75 mM free 3HPA (■)], at a rate of

0.16 mL/min in a downward direction over a column packed with 1 g of Amberlite IRA-400 (Cl). The data represent the mean of two independent replicates \pm SD.

Fig. 4. Chromatographic profiles of 3HPA-bisulfite complex (\blacklozenge), free 3HPA (\blacksquare) and total 3HPA (\blacktriangle) on loading 5 mL solution containing 61.08 ± 7.18 mM 3HPA-bisulfite complex and 49.41 ± 3.66 mM free-3HPA over 1.0 g Amberlite IRA-400 (Cl) packed in a column, followed by washing with deionized water, and elution with 0.20 M NaCl. The data represent the mean of two independent replicates + SD.

Fig. 5. Gas chromatogram of (A) 4 times diluted sample of the reaction mixture containing 3HPA (106.22 ± 0.31), 1,3PDO (27.6 ± 0.00 mM), and remaining glycerol (65.2 ± 0.00 mM) prior to passing over the column of Amberlite IRA-400 (Cl) resin, (B) 3HPA containing solution eluted from the resin by 0.20 M NaCl, and (C) unbound fraction containing 1,3PDO, glycerol and very low amount of 3HPA.

Fig. 6. (A) Production of 3HPA from glycerol using *L. reuteri* in a fed-batch system with *in situ* 3HPA complexation using sodium bisulfite and separation through binding to 50.0 g Amberlite IRA-400 (SO₃H). The symbols indicate the concentrations of residual glycerol (\blacklozenge), 1,3PDO (\blacktriangle), 3HP (\blacksquare), free 3HPA (\times), 3HPA-bisulfite complex (\ast), and total 3HPA (\bullet). (B) Kinetics of glycerol consumption (\blacksquare) and 3HPA production (\bullet) for the same process. The data represent the mean of two independent replicates \pm SD.

Scheme 1. Biotransformation of glycerol (1) to 3HPA (2) and further to 1,3PDO (3) and 3HP (4) by *L. reuteri*, 3HPA-bisulfite complex (5) formation, binding of the complex to an ion exchange resin (6) and elution of the complex and free aldehyde using sodium chloride.

Fig. 1.

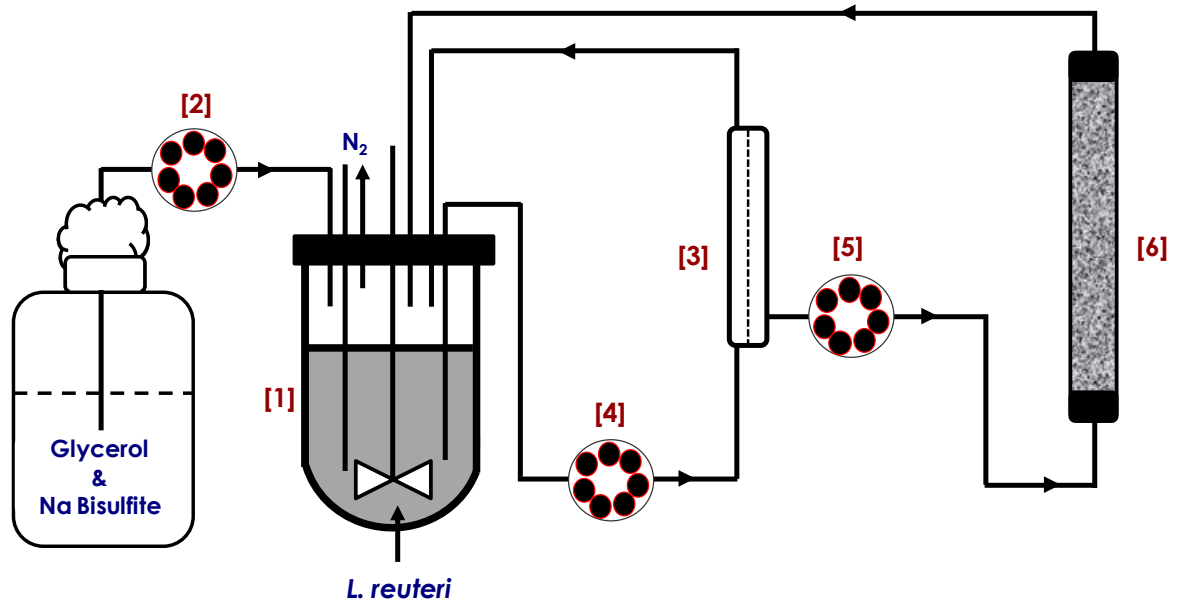


Fig. 2.

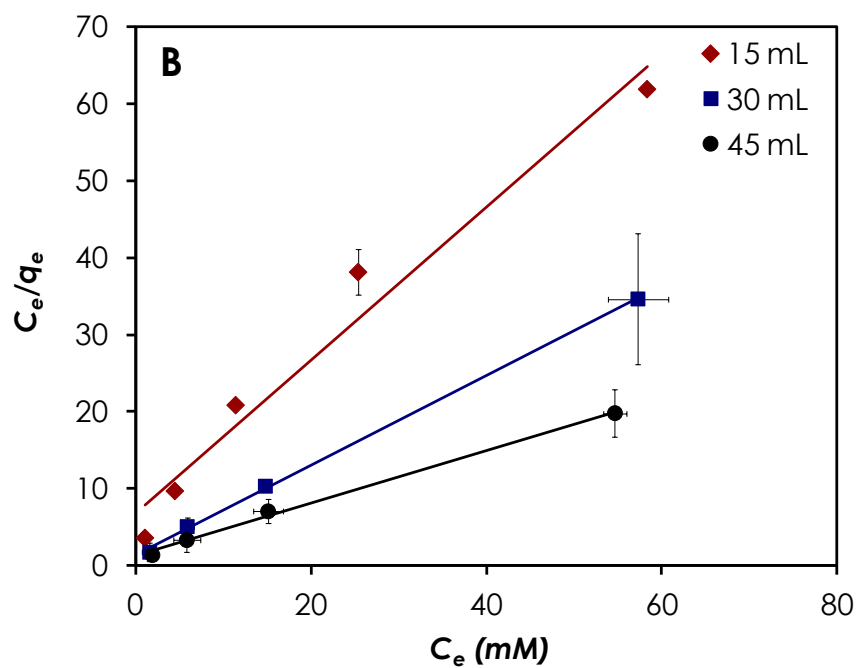
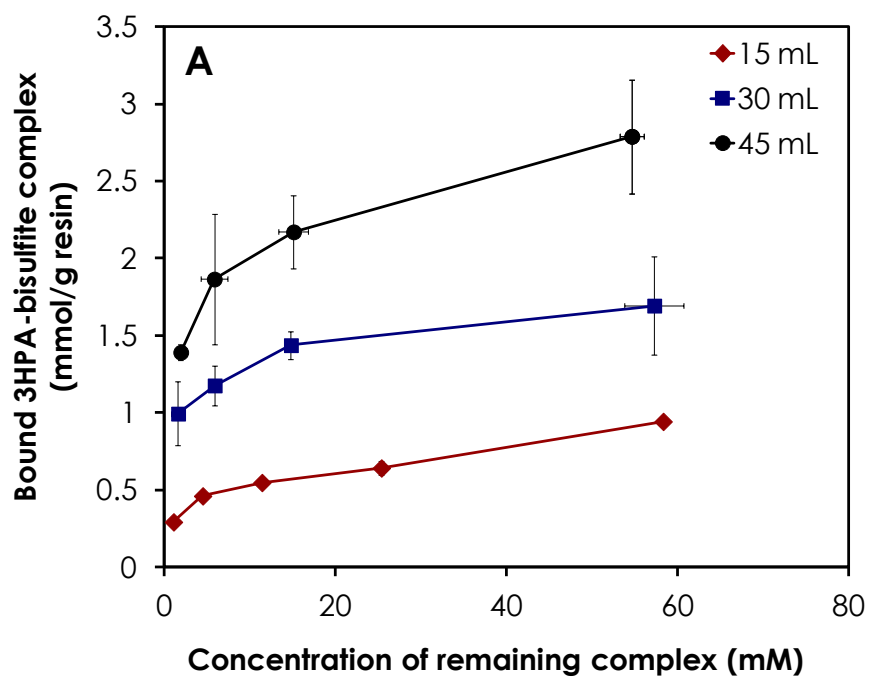


Fig. 3.

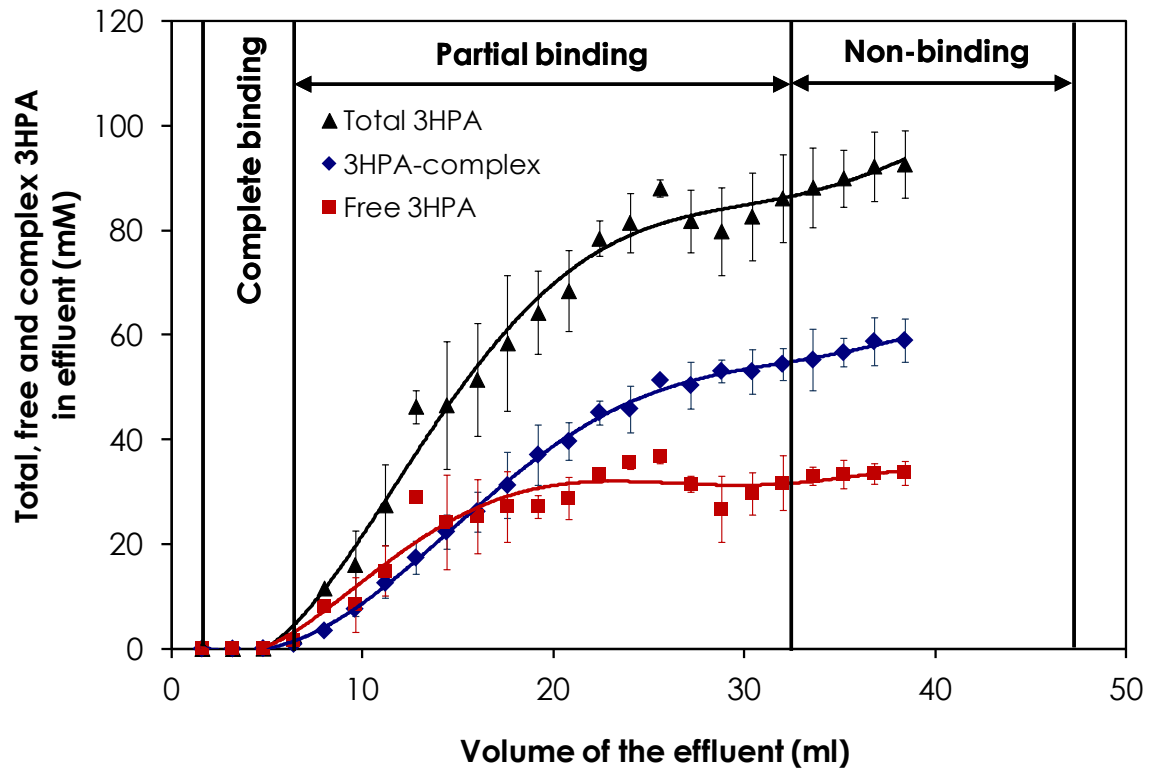


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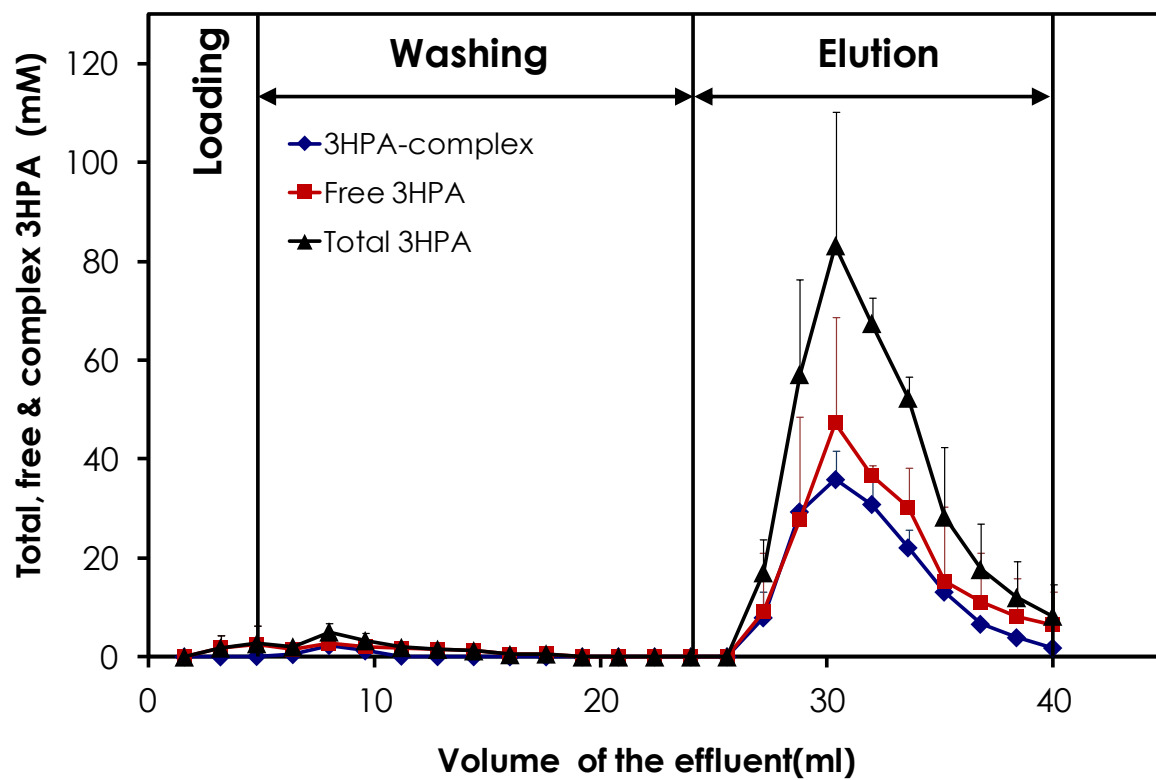


Fig. 5.

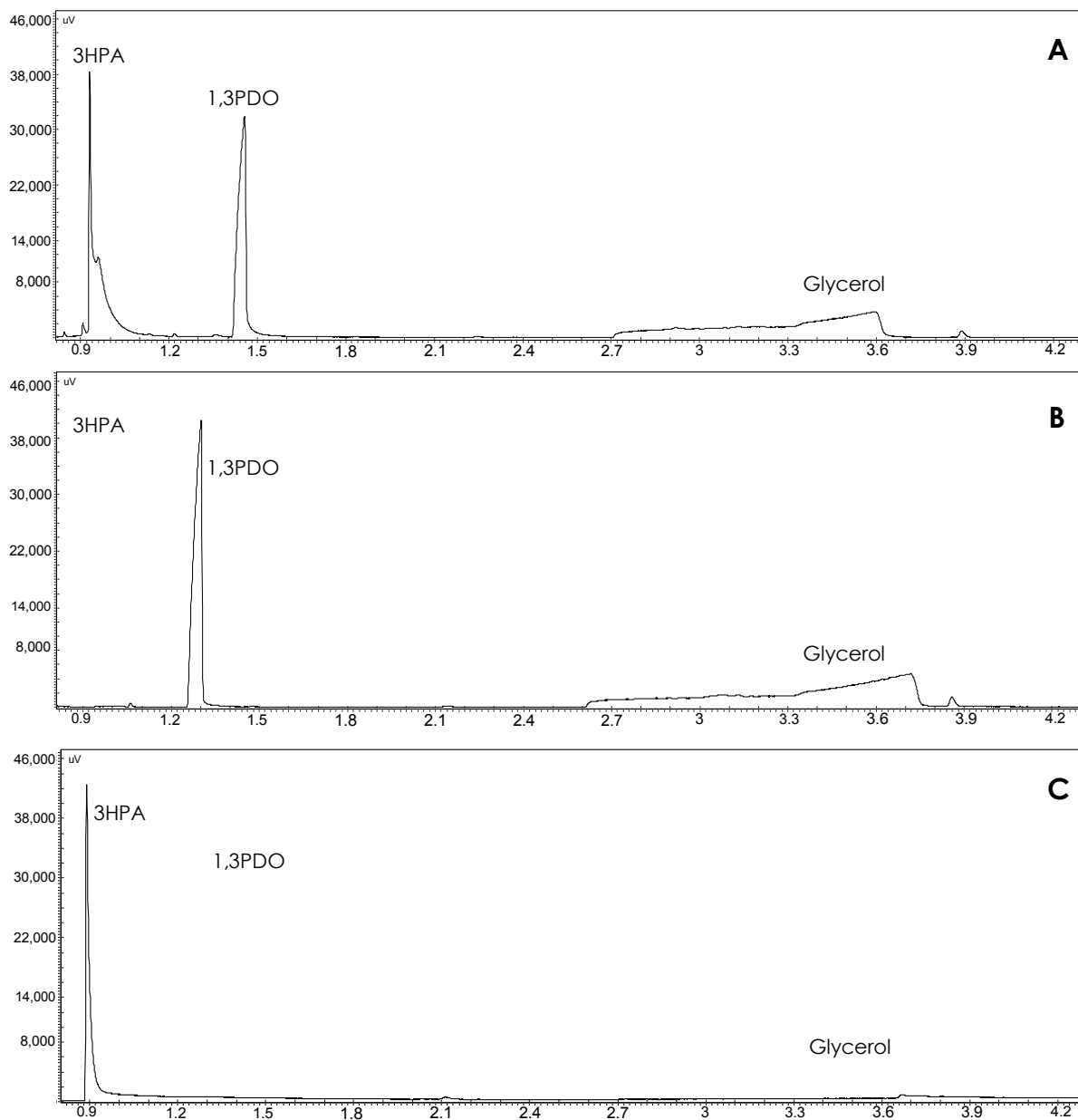
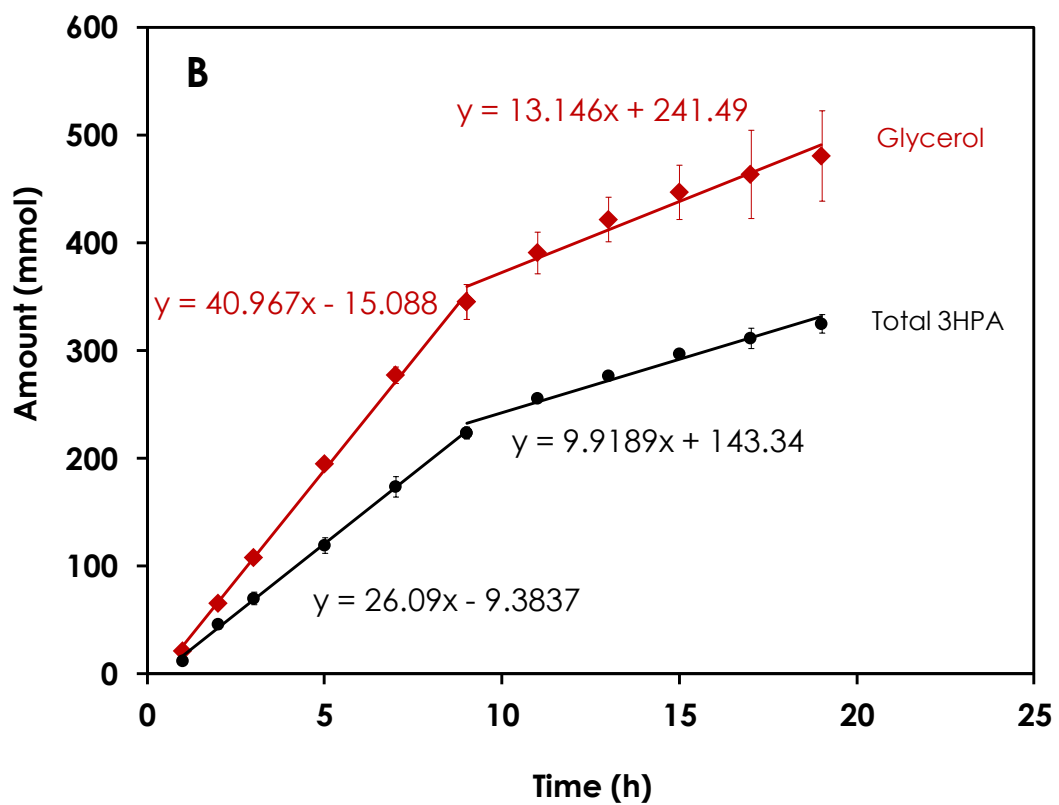
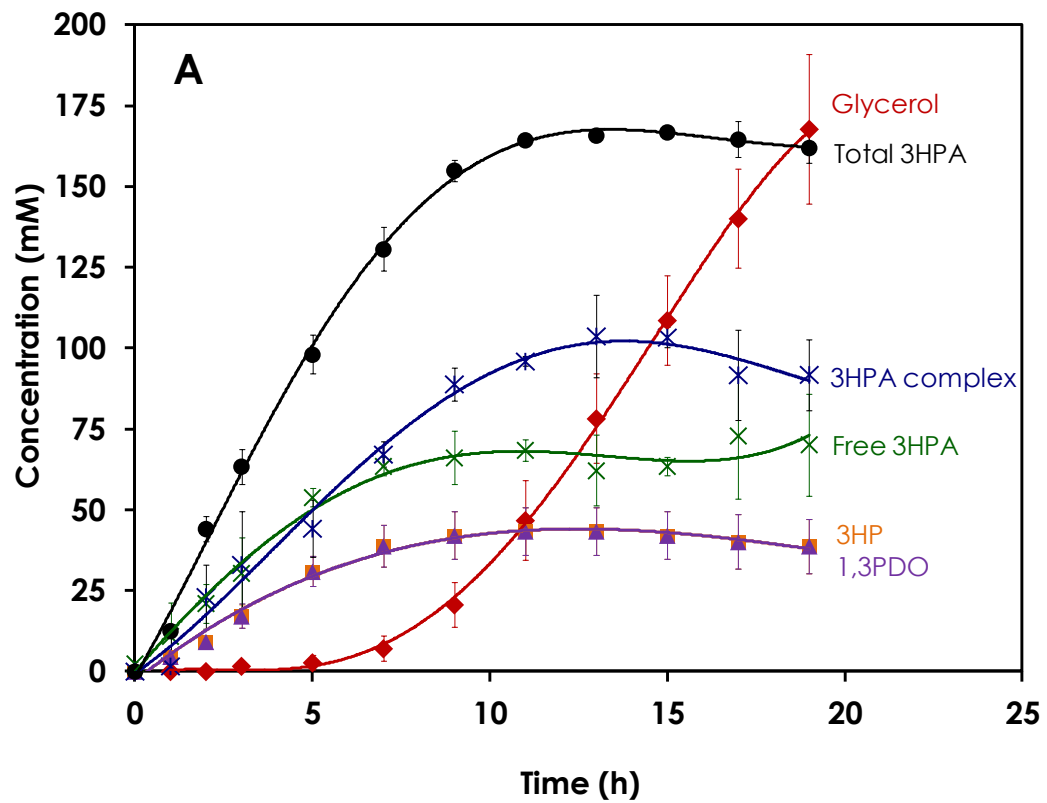


Fig. 6.



Scheme 1.

