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Rezaei, Roya; Dishisha, Tarek; Pyo, Sang-Hyun; Hatti-Kaul, Rajni

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PO Box 117
221 00 Lund
+46 46-222 00 00

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Title: Semicarbazide-functionalized resin as a new scavenger for *in-situ* recovery of 3-hydroxypropionaldehyde during biotransformation of glycerol by *Lactobacillus reuteri*

Author: Roya R.R. Sardari Tarek Dishisha Sang-Hyun Pyo
Rajni Hatti-Kaul



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Highlights

- Semicarbazide-functionalized resin, a novel non-toxic scavenger for 3HPA
- Semicarbazide-resin exhibits high binding capacity for 3HPA
- 3HPA recovery from the resin by sequential elution with water and acetic acid
- *In situ* recovery of 3HPA using the resin gives improved production

Semicarbazide-functionalized resin as a new scavenger for *in-situ* recovery of 3-hydroxypropionaldehyde during biotransformation of glycerol by *Lactobacillus reuteri*

Roya R. R. Sardari, Tarek Dishisha, Sang-Hyun Pyo and Rajni Hatti-Kaul*

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Box 124, SE-221 00 Lund, Sweden

** Corresponding author:*

E-mail: Roya.Rezaei@biotek.lu.se

Tel. +46-46-222 8157 Fax. +46-46-222 4713

Abstract

3-Hydroxypropionaldehyde (3HPA), a potential C3-platform chemical for a biobased industry, is produced from glycerol using *Lactobacillus reuteri* through its glycerol dehydratase activity. However, the process is characterized by low yield and - productivity due to toxic effects of 3HPA on the biocatalyst activity. In this study, a semicarbazide-functionalized resin was prepared, evaluated for adsorption and *in situ* recovery of 3HPA during biotransformation of glycerol. Adsorption of 3HPA onto the resin was characterized as “*S-curve* model”, increasing with increasing initial 3HPA concentration, and reached a maximum of 9.48 mmol/g_{resin} at 71.54 mM 3HPA used. Desorption of 3HPA was evaluated using water and different acids, and was enhanced by acetic acid with organic modifiers. Repeated adsorption-desorption of 3HPA in batch resulted in elution of 13-66.5% of the bound 3HPA during at least three sequential cycles using water and acetic acid, respectively as eluants. Using the resin for *in situ* product removal led to more than 2 times higher productivity of 3HPA.

Keywords: 3-Hydroxypropionaldehyde; *Lactobacillus reuteri*; Semicarbazide-functionalized resin; Whole cell biotransformation; *In situ* product removal

1. Introduction

3-Hydroxypropionaldehyde (3HPA) has attracted attention during recent years as a potential platform chemical for biobased production of 1,3-propanediol (1,3PDO), acrolein, 3-hydroxypropionic acid (3HP) and acrylic acid (Vollenweider et al., 2003; Vollenweider and Lacroix, 2004). Dehydration of glycerol, a renewable co-product of biodiesel production, catalyzed by glycerol dehydratase using whole cells of *Lactobacillus reuteri* as a biocatalyst, is a promising method for production of 3HPA (Ulmer and Zeng, 2007; Vollenweider and Lacroix, 2004). However, in spite of tolerating relatively high concentrations of the hydroxyaldehyde, the enzyme activity and viability of *L. reuteri* are negatively affected at 3HPA concentration higher than 30-50 mM and 60-120 mM, respectively (Cleusix et al., 2007). Removal of 3HPA from the vicinity of the cells to increase the lifetime of the biocatalyst, and consequently the 3HPA yield and productivity, is essential.

Carbonyl compounds undergo an addition-elimination reaction with amines to form stable imines (C=N). The reaction occurs in two steps, the first step is the *addition* of the nucleophile (amine) to the carbonyl group yielding an intermediate, which subsequently forms a highly stable imine by *elimination* of a water molecule. The first step is the rate-limiting step with most of the amines. However, when very basic amines such as semicarbazide or aniline are used, the “*elimination*” is considered the rate-limiting step (Carey, 1987), and can be accelerated by using concentrated acids or thermal treatment (Wild, 2003). Making use of this classic addition-elimination reaction with aldehyde, semicarbazide and carbohydrazide have been used as scavengers to form 3HPA-semicarbazide intermediate and carbohydrazone, respectively, and in turn

resulting in considerably enhanced productivity of 3HPA (Krauter et al., 2012; Ulmer and Zeng, 2007). Further thermal treatment of the resulting 3HPA-semicarbazide intermediate at 90 °C resulted in precipitation of the highly stable semicarbazone (Ulmer and Zeng, 2007) (Scheme 1A).

Although resulting in high 3HPA titers and production rates, these complexation methods shared the common problem of low 3HPA purity, difficult separation of 3HPA complexes from the solution, and the hydrolytic cleavage of the resulting imines to release the free aldehyde is not feasible and requires harsh conditions.

Hydrolytic cleavage of semicarbazones using aqueous acids (Bhar and Guha, 2005) and gaseous nitrogen dioxide (Naimi-Jamal et al., 2012) has been reported for the recovery of the free aldehydes. However, the recovery of 3HPA from the corresponding semicarbazone and carbohydrazone has not yet been reported. While the semicarbazones are highly stable, the cleavage of the less stable intermediate could be done relatively easier to yield 3HPA (Wild, 2003). Furthermore, selective binding of the 3HPA-semicarbazide intermediate to a resin and subsequent release of the free aldehyde can guarantee higher purity of the desired product.

Preparation and development of a semicarbazide-functionalized resin has been reported for solid-phase preparation of peptide ketones and aldehydes (Vázquez and Albericio, 2006). The aim of the present study was to evaluate the semicarbazide-functionalized resin as a matrix for adsorption of 3HPA to form the intermediate as shown in Scheme 1, and as a potential method for *in situ* recovery of 3HPA during its production from glycerol.

2. Materials and methods

2.1 Materials

(Aminomethyl)polystyrene resin (5 g package), N,N-dimethylformamide, 1,1'-carbonyldiimidazole, dichloromethane, *tert*-butyl carbazate, glycerol, 1,3-propanediol and DL-tryptophan were procured from Sigma-Aldrich (MO, USA). Acetic acid (100 %) was obtained from BDH Prolabo (VWR International, Fontenay-sous-Bois, France), and 3-hydroxypropionic acid (30% w/v) was provided by Perstorp AB, Sweden. 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 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g Tween 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate and 2 g dipotassium phosphate) was a product of Difco (MD, USA).

2.2. Biocatalyst production

The resting cells of *L. reuteri* DSM 20016 (DSMZ culture collection, Germany) were used for 3HPA production from glycerol. Twenty milliliter of the culture medium (55 g/l MRS containing 20 mM glycerol) in 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. Twenty milliliter of this culture was used as inoculum for 2 l culture medium (55 g/l MRS containing 20 mM glycerol) in the 3 l bioreactor (Applikon, The Netherlands). Fermentation conditions chosen were pH 5.5, temperature of 37 °C, stirrer speed of 200 rpm, and continuous bubbling with N₂ gas. The fermentation was stopped after 8 hours and the cells were harvested by centrifugation of the broth at 10,000 × g and 4 °C for 5 min using Sorvall

centrifuge (RC5C, USA). The cell pellet was washed once by re-suspension in 50 ml of 50 mM sodium acetate buffer pH 5 and then re-centrifuged.

2.3. 3HPA production from glycerol in batch and fed-batch modes of biotransformation

3HPA was produced by batch and fed-batch biotransformation of glycerol as described elsewhere (Sardari et al., 2013b; Ulmer and Zeng, 2007). In the batch mode, *L. reuteri* cells were re-suspended in 197.11 ± 0.76 mM glycerol solution and incubated at 37 °C under anaerobic conditions. After 2 h, the cells were separated by centrifugation at $10,000 \times g$ and 4 °C for 5 min and the supernatant obtained contained 85.41 ± 3.40 mM 3HPA from the reaction with 5 g cell dry weight and 107.12 ± 1.27 mM 3HPA with 7.5 g cell dry weight.

In the fed-batch biotransformation, the fresh cells (5 g cell dry weight) were re-suspended in 21.72 mM glycerol solution at pH 5 and 37 °C. After 1 hour of batch biotransformation, 300 ml of feeding solution containing 50 g glycerol was fed at a rate of 1 ml/min. The experiment was stopped after 6 hours and the supernatant was separated from the cells. From two separate fed-batch biotransformation, solutions with two different concentrations of 3HPA $48.70 (\pm 0.53)$ mM and $71.54 (\pm 1.70)$ mM at pH 5 were obtained, which were used for further studies.

2.4. Preparation of semicarbazide-functionalized resin

The semicarbazide-functionalized resin was prepared from the aminomethyl polystyrene resin (Vázquez and Albericio, 2006). Two grams of aminomethyl polystyrene resin was weighed into a 50 ml glass tube, and swelled in N,N-dimethylformamide (DMF) for 24

h. A solution of N,N'-carbonyldiimidazole (CDI) in DMF (437 mM) was added to the resin suspension to activate the aminomethyl group on the resin. The mixture was shaken at room temperature for 3 h, after which the resin was washed three times with DMF. A solution of *tert*-butyl carbazate in DMF (437 mM) was then added to the resin and the mixture was shaken at room temperature for 3 h followed by washing 3 times each with DMF and dichloromethane (DCM). The resin was left overnight to dry. Before using the resin, it was treated with trifluoroacetic acid (TFA)-DCM (1:1) solution for 1 h at room temperature, and then washed 3 times each with DCM, ethanol and water, respectively.

2.5. Static binding capacity of the functionalized resin in batch and continuous systems

Fifty milligram of the functionalized resin was mixed with 10 ml 3HPA solution prepared in Section 2.3 containing 71.54 ± 1.70 mM 3HPA in a 20 ml glass tube, and the liquid samples were collected after 0.5, 3, 6, 18, 24, 30, 42 and 48 hours. A parallel experiment was done under the same conditions and samples were taken every 5 min for 30 min. Samples were analyzed for the concentration of unbound 3HPA from which the amount of 3HPA bound per gram resin was calculated.

The static binding capacity in batch system and adsorption isotherm were determined for semicarbazide resin (0.05 g dry weight) by equilibrating with 50 mL of freshly prepared 3HPA with concentration ranging between 9.67 ± 0.48 mM and 71.54 ± 1.70 mM for 48 h. Samples were collected and analyzed for the concentration of unbound 3HPA at equilibrium and the amount of 3HPA bound per gram resin was calculated for each solution and concentration.

Two chromatographic columns (0.5 cm internal diameter \times 20 cm height) packed with 0.5 g functionalized resin were used for determination of breakthrough curve and static binding capacity in continuous system. Twenty five milliliters of the supernatant from the bioconversion experiment containing 48.70 ± 0.53 mM and 107.12 ± 1.27 mM 3HPA, respectively, were passed through the columns at a flow rate of 0.1 ml/min, respectively. The effluents were collected in fractions of 1 ml per 10 min using a fraction collector (LKB Bromma, 2212 Helirac, Sweden) and the concentration of 3HPA was measured in the fractions. The solution with 48.70 ± 0.53 mM 3HPA was also passed at a flow rate of 0.036 ml/min over the resin and the effluents were collected in fractions of 1.08 ml per 30 min and the concentration of 3HPA was measured.

2.6. Elution and separation of 3HPA

To eleven 20 ml glass tubes, each containing 0.2 g resin, 10 ml of 3HPA solution (48.70 ± 0.53 mM) was added and shaken at room temperature for 30 min. After separation of solution, elution of the bound 3HPA was studied by treating the resin: (i) first with 5 ml deionized water (3 times), and then (ii) with 5 ml of acetic acid at different concentrations, 0.25, 0.75, 1.5, 3, 5, 7, 9, 11, 13, 15 M and glacial, respectively. Liquid samples were taken after 0.5, 2, 4, and 6 hours. For the 2nd elution, hydrochloric acid (37 %), TFA (99 %) and phosphoric acid (85 % wt. in H₂O), respectively, were evaluated and compared with acetic acid for their ability to release 3HPA. Elution was also studied with 3 M acetic acid mixed with an organic modifier; DMF, ethanol and methanol, respectively, with water at a ratio of 1:1. The concentration of 3HPA was measured in the samples during loading, washing and elution steps.

2.7. Repeated batch adsorption-desorption of 3HPA

The adsorption-desorption experiments were carried out using 48.70 ± 0.53 mM and 85.41 ± 3.41 mM 3HPA, respectively, in 20 ml glass tubes. In the first batch, 0.2 g resin and 10 ml 3HPA solution were added to each tube, and equilibrated for 30 min and 12 hours, respectively. After removal of the supernatant, elution of 3HPA was studied by treating the resin first with 5 ml deionized water (3 times), and then with 5 ml acetic acid (9 M) for 2 hours. This was followed by washing the resin with 5 ml deionized water (3 times) to make it ready for the next batch. The second and third batches were repeated in the same way as the first batch, but using the supernatant from the previous batch for adsorption. Samples were taken and 3HPA concentration was measured in each step.

2.8. Batch biotransformation of glycerol with *in situ* 3HPA removal

L. reuteri cells (0.5 g wet weight) prepared as described in Section 2.2 were resuspended in 10 ml of 200 mM glycerol solution in a 50 ml sterile Falcon tube. The tube was incubated with mixing on a rocking table at 37 °C under anaerobic conditions. Three different experiments were performed by including 0, 0.5, and 1 g semicarbazide-functionalized resin, respectively, in the tubes. Samples were collected after 3 and 24 h and the concentrations of 3HPA, 1,3PDO, 3HP and residual glycerol were measured.

The total 3HPA (free and bound to the resin) and percent 3HPA bound to the resin were calculated using the following equations:

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

$$\text{3HPA bound to the resin (\%)} = [(\text{Total 3HPA} - \text{3HPA in solution}) / (\text{total 3HPA})] * 100 \quad (\text{Eqn 2})$$

2.9. Quantitative analyses

The colorimetric method of Circle et al. (1945) as modified by Ulmer and Zeng (2007) was used for measuring total 3HPA (reuterin) concentration using acrolein as standard. The assay is based on dehydration of 3HPA using concentrated hydrochloric acid to acrolein that reacts with DL-tryptophan to give a purple color. Briefly, 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 then incubated at 37 °C for 20 min and the resulting purple color was measured at 560 nm using an UV/Vis spectrophotometer (Ultrospec 1000, Pharmacia biotech, Sweden).

The concentrations of glycerol, 1,3PDO and 3HP were determined by HPLC (JASCO) on an Aminex HPX-87H chromatographic column, connected to a guard column (Biorad, Richmond, CA, USA), and maintained at 65 °C using a column oven (Shimadzu). The properly diluted samples from the bioreactor were mixed with 20% v/v sulfuric acid (20 µl/ml of sample) and then filtered. Forty microliter of the sample was injected in a mobile phase of 0.5 mM H₂SO₄ flowing at a rate of 0.4 ml/min. All the compounds were detected using RI-detector (ERC, Kawaguchi, Japan), and 3HP concentration was confirmed by detection at 215 nm using a UV detector (Jasco).

All the experiments in Sections 2.5 - 2.8 were performed in two independent replicates. The data are presented as the mean of the replicates ± standard deviation.

3. Results and discussion

3.1. Evaluation of 3HPA binding to semicarbazide-functionalized resin

The semicarbazide-functionalized resin was prepared by activating the aminomethyl polystyrene resin with carbonyldiimidazole and then treating with *t*-butyl carbazate. It was then evaluated for batch adsorption of 3HPA from the aqueous solution with initial concentration of 71.54 ± 1.70 mM (pH 5) (Fig. 1). The amount of 3HPA remaining in the supernatant was determined every 5 min for the initial 30 min and less frequently up to 48 h. The results show high affinity of the semicarbazide ligand for binding 3HPA molecules. A rapid uptake of 3HPA from the solution was observed as the adsorbed amount reached 2.10 mmol/g resin within the initial 5 min, and was increased to 2.59 mmol/g resin at 30 min (Fig. 1A). The amount of 3HPA adsorbed reached a maximum of 4.15 ± 0.68 mmol/g resin (38% of the loaded amount) after 24 h (Fig. 1B), and was constant thereafter.

3HPA is present in a concentration-dependent equilibrium mixture of 3HPA monomer (1), hydrate (1a) and dimer (1b), (Scheme 1C) (Vollenweider et al., 2003). The equilibrium is established very fast; according to Vollenweider et al. (2003), the three 3HPA forms are in equilibrium within the time needed for sample preparation, i.e. 15 min. Studies performed on kinetics of equilibrium attainment for a similar compound, glycolaldehyde, indicate that transformation of the isomeric structures into each other occurs in milliseconds (Glushonok et al. 2000). The monomer is the only form with a carbonyl group which can undergo rapid interaction with the amine group of the semicarbazide ligand. At the operating concentration (≤ 0.1 M), the hydrate, dimer and monomer forms represent about 70, 4, and 25%, respectively, of the HPA system (Vollenweider et al., 2003). This was comparable with the percent 3HPA (25%) that has been bound from the total loaded amount within the initial 30 min (Fig. 1A).

The binding capacity of 3HPA to the semicarbazide-functionalized resin was 4.4 and 9.6 times, respectively higher than that on the native aminomethyl polystyrene resin and the pre-activated resin (after swelling in DMF and treatment with CDI), respectively, within the initial 30 min (2.5 vs. 0.57 and 0.26 mmol/g resin, respectively). This characteristic fast binding is highly desirable for *in situ* removal of 3HPA during biotransformation of glycerol to protect the cells from the toxic effects of 3HPA.

3.2. Determination of maximum resin capacity and adsorption isotherm

The binding capacity of the resin was then investigated using 50 ml solution with varying 3HPA concentration in the range of 9.67 (± 0.48) mM to 71.54 (± 1.70) mM (pH 5) using a batch equilibrium method. Generally, by increasing the initial 3HPA concentration the amount adsorbed to the resin was increased. For concentrations of 3HPA below 40 mM, the amount of 3HPA adsorbed ranged between 5-5.78 % of the initial loading. At higher concentrations (49.81 \pm 4.40 and 71.54 \pm 1.70 mM), the percent 3HPA bound was increased to 11.82 % (5.88 \pm 0.53 mmol/g resin) and 13.26 % (9.48 \pm 0.00 mmol/g resin), respectively. This indicated that 3HPA adsorption/interaction with the semicarbazide is promoted at higher concentrations. The maximum binding capacity of the resin for 3HPA molecules was 9.48 (± 0.00) mmol/g resin for the initial concentration of 71.54 (± 1.70) mM 3HPA under the experimental conditions which shows the high affinity of resin for adsorption of 3HPA.

A curve correlating the amount of 3HPA bound to the resin and the concentration of residual 3HPA in solution was employed for identification of the adsorption isotherm (Fig. 2). According to the initial slope and the overall pattern of the curve, 3HPA binding to the semicarbazide-functionalized resin can be best described according to the

“*S-curve* model”, which is subgroup “3” in the classification of solution adsorption isotherms by Giles et al. (1960). An initial gradual increase in binding capacity with increasing 3HPA concentration from $9.67 (\pm 0.48)$ mM to $18.97 (\pm 1.50)$ mM was observed. This increase was followed by a partial plateau and finally a rapid increase in the binding capacity when the initial 3HPA concentration exceeds $30.48 (\pm 1.40)$ mM.

The “*S-curve*” adsorption isotherm occurs in rare cases such as adsorption of surfactants on hydrophilic surfaces, aromatic hydrocarbons on calcined alumina, non-polar organic compounds on clay, and metal complexation and adsorption to a matrix with soluble ligand (Connaughton et al., 1993; Giles et al., 1960; Mittal and Shah, 2002; Sanchez-Martin et al., 2006; Sposito et al., 1999). Generally, this type of adsorption takes place when the binding of the solute to the matrix occurs via two different mechanisms. The first is mostly the complexation occurring between the carbonyl group of 3HPA and the semicarbazide ligand, “Chemisorption” (Sattler and Feindt, 2007).

The short plateau indicates a first degree saturation of the semicarbazide-ligands on the resin surface (Giles et al., 1960). The bound 3HPA at the plateau (1.75 ± 0.01 mmol/g resin) was in agreement with the reported theoretical binding capacity of the polystyrene resin (1.5-3 mmol/g resin) provided by the supplier. The 3HPA-adduct will form a hydrophilic layer around the resin which facilitates the adsorption and/or intermolecular attraction with other 3HPA molecules from the solution at higher 3HPA concentration, “physical adsorption/interaction”. This is known as co-operative adsorption (Giles et al., 1960).

3.3. Determination of maximum binding capacity in continuous adsorption system and breakthrough curve

Fig. 3 shows the breakthrough curve of 3HPA on passing 25 ml 3HPA containing solution through a column packed with 0.5 g semicarbazide-functionalized resin. Two different concentrations of 3HPA (107.12 ± 1.27 mM and 48.70 ± 0.53 mM) were evaluated at a flow rate of 0.1 ml/min. Binding of the entire 3HPA from the solution was observed for the initial 3 ml and was followed by increase in the concentration of 3HPA in the effluents until 6 ml, and reaching a plateau till 25 ml. The adsorption of 3HPA results in re-establishment of the equilibrium with the hydrate and dimer forms being converted to the monomer that binds to the resin until the maximum capacity is reached.

The maximum binding capacity was almost doubled at higher initial 3HPA concentration (1.30 vs. 0.71 mmol/g resin). The concentration of 3HPA in the effluent at the plateau corresponded to 90% of the initial loading concentration. At the 3HPA concentration of 48.70 ± 0.53 mM, reducing the flow rate from 0.1 to 0.036 ml/min resulted in 44% increase in the binding capacity and the 3HPA concentration of the effluent at the plateau corresponded to 77% of the initial load.

The obviously low static binding capacity compared to the batch mode indicates that only a single mode of binding “chemisorption” might be prevalent. Hence, the maximum capacity of resin in continuous adsorption might be increased by recirculation of the 3HPA solution through the column. Compared to the breakthrough curve of 3HPA-bisulfite complex on Amberlite IRA-400 (Cl) reported earlier (Sardari et al., 2013a), the semicarbazide-functionalized resin showed a steeper slope with 35% lower static binding capacity. However, the batch binding capacity was 3 times higher.

3.4. Elution and separation of 3HPA

Since semicarbazide and carbohydrazide complexes with 3HPA result in significant increase in 3HPA productivity and biocatalyst activity, a successful dissociation of the resulting complexes for releasing the free aldehyde is highly desirable. To our knowledge this step has not been reported earlier for 3HPA complexes. There are many studies for breakdown of semicarbazones and release of free aldehyde using various reagents such as nitrous oxide, trifluoroacetic acid (TFA), calcium hypochlorite and moist montmorillonite K-10 (Baruah et al., 1998; Bhar and Guha, 2005; Movassagh and Dahaghin, 2006; Ram and Varsha, 1991). However, these strategies are active mostly with cyclic carbonyl compounds or include reagents that are highly toxic, corrosive, and polluting. Besides the emergence of undesired side reactions (Bhar and Guha, 2005), the recovery of the aldehyde from these chemicals could be economically unfeasible.

In the present study a number of acids including hydrochloric acid (HCl), TFA, phosphoric acid, and acetic acid were evaluated for their ability to release 3HPA. Using concentrated acids resulted in poor elution with recovery of 1.14, 2.13, 0.85 and 1.13%, respectively, which could be due to the concentrated acid-mediated dehydration of 3HPA-semicarbazide intermediate (HO-C-N-H) to the highly stable semicarbazone (-C=N-), and of 3HPA to acrolein (Circle et al., 1945; Jencks, 1959). Since TFA and HCl are highly corrosive, acetic acid was chosen for the further experiments.

The semicarbazide ligand is attached to an aromatic benzene ring on the resin, which might shield the access of the hydrophilic acidic reagents to the 3HPA-semicarbazide intermediate. Hence, the effect of different organic modifiers in 3 M acetic acid on the elution of 3HPA was investigated. The recovery of 3HPA determined after 2 h elution time was increased from 10% with 3 M acetic acid to 15.29 %, 20.24 %, and 17.60 % in the presence of 50% (v/v) DMF, ethanol and methanol, respectively.

Also, the effect of acetic acid concentration on 3HPA elution from the semicarbazide resin was investigated at room temperature and 95 °C, respectively. While poor elution was obtained at low acetic acid concentration (0.25, 0.75 and 1.5 M), the eluted amount of 3HPA was doubled with increase in acetic acid concentration to 7-13 M, but with further increase in the acetic acid concentration to > 13 M the amount of eluted 3HPA was reduced. The maximum 3HPA recovery from the resin was 16% after 2 h elution with 9 M acetic acid (Fig. 4A) which was close to the recovery in the presence of organic modifiers. Hence, 9 M acetic acid was chosen to reduce the purification steps. The total 3HPA recovery by 1st elution using deionized water and 2nd elution using acetic acid (9M) was 55.67 % (Fig. 4B). At higher temperature (95 °C), the elution of 3HPA was very poor.

3.5. Repeated adsorption-desorption of 3HPA in batch mode

The semicarbazide-functionalized resin was evaluated for the ability of repeated adsorption-desorption of 3HPA over three subsequent cycles, each cycle consisting of loading, 1st elution with deionized water, 2nd elution with 9 M acetic acid and washing with deionized water to remove acetic acid. Four parallel experiments were performed with varying adsorption time (30 min or 12 h), as well as the concentration of 3HPA in the loading solution during the first cycle (48.70 ± 0.53 or 85.41 ± 3.41 mM). In each subsequent cycle, the solution from the previous cycle containing the unbound residual 3HPA was used as the loading solution.

Table 1 shows the amount of 3HPA adsorbed, and eluted in each cycle. In general, longer adsorption time and higher initial concentration of 3HPA resulted in higher adsorption. Considering the effect of the initial 3HPA concentration in the adsorption

step, the amount of 3HPA adsorbed was increased at higher concentration (Table 1), which followed the adsorption isotherm as a “*S-curve* model” consisting of “Chemisorption” and “physical adsorption/interaction” in Fig. 2.

The amount of 3HPA eluted from the resin with water was also increased about 1.4 – 2 times in the batches using the higher initial 3HPA load. Interestingly, higher amount of 3HPA was recovered from the resin involving shorter adsorption time (30 min) than that for 12 h adsorption although the amount of 3HPA adsorbed was less during 30 min. Also, the amount of 3HPA bound and eluted decreased in the order of cycle#1 > cycle#2 > cycle#3, that may be due to saturation of the resin and reduction of 3HPA concentration in the loading solution in the second and third batches, which plays a major role in the mechanism of adsorption. It is noteworthy that in the experiment involving 85.40 mM 3HPA and binding for 30 min, the elution of the bound aldehyde was nearly quantitative during 2nd and 3rd cycle. Hence, the release of 3HPA seems to be easier from low density adsorbed layer formed during 30 min on semicarbazide-functionalized resin than from the high density of 3HPA packed for longer time, which would be the second stage of isotherm, “physical adsorption/interaction” in Fig. 2.

The total 3HPA eluted over the three cycles varied in the range of 13 - 66.5% of the bound 3HPA.

3.6. *In situ* 3HPA removal during biotransformation of glycerol

The semicarbazide-functionalized resin was then employed for *in situ* capture of 3HPA during biotransformation of glycerol by resting cells of *L. reuteri* (0.5 g wet weight) as biocatalyst. The production was evaluated in the presence of two different quantities of

the resin (0.5 and 1 g), and the results were compared with a control experiment without resin.

Fig. 5 shows the concentration of consumed glycerol and total 3HPA produced (free in solution and bound to the resin) after 3 h of biotransformation. Low cell density was chosen to avoid rapid accumulation of the aldehyde to toxic levels. As a result, in all cases partial consumption of the supplied glycerol was observed. 3HPA was the main product produced with 1,3PDO and 3HP as by-products.

In case of production without *in situ* recovery, a total of 21.04 (\pm 7.56) mM 3HPA was produced in 3 h at a rate of 7.01 mM/h. The produced 3HPA represented 42 mol% of the consumed glycerol. Addition of 0.5 g resin to the reaction resulted in enhanced 3HPA production at concentration of 32.44 ± 0.65 mM, volumetric productivity of 10.81 mM/h, and molar yield of 53 mol%, respectively. Increasing the amount of the resin to 1 g was accompanied by 50% and 128% increase in total 3HPA concentration and volumetric productivity over that obtained with 0.5 g resin and with the control, respectively. Even the 3HPA yield was increased to 59 mol% caused by reduced metabolic flux towards 3HP and 1,3PDO. In the presence of the resin, the concentration of the free 3HPA in the solution represented a minor fraction (2-3% of the total 3HPA produced), indicating the high efficiency of the resin as 3HPA scavenger (Table. 2).

In contrast to Amberlite IRA-400 and free semicarbazide, the semicarbazide-functionalized resin does not show any inhibitory effect on 3HPA production (Rütti et al., 2011). The production of 3HPA was increased as a result of reduced 3HPA toxicity to the biocatalyst, limited formation of by-products, and maintained a longer biocatalyst activity (data not shown).

Further work will involve developing a better method for preparation of the functionalized resin with minimal use of volatile, chlorinated solvents, and further evaluating the integration of the semicarbazide-resin with fed-batch biotransformation of glycerol on productivity and biocatalyst lifetime.

4. Conclusion

This paper demonstrates the application of the semicarbazide-functionalized resin for adsorption of 3HPA, and as a promising alternative to the methods reported so far for *in situ* removal of 3HPA during its production from glycerol using *L. reuteri*. The only means of recovering 3HPA reported so far have been complexation in solution with semicarbazide or carbohydrazide that should be heated at 100 °C to get 3HPA semicarbazone precipitate. Moreover, the cleavage of 3HPA semicarbazones needs extremely harsh conditions.

By using the functional group bound to a resin allows much easier separation of 3HPA from biotransformation by-products and residual glycerol. This was demonstrated earlier by us by using bisulfite-functionalized Amberlite 400 resin (Sardari et al., 2013a). The semicarbazide-functionalized resin exhibits significantly higher rate of adsorption and -capacity for binding 3HPA than the bisulfite resin. It was shown that although the binding capacity was sacrificed by reducing the time for adsorption, it was significantly easier to recover 3HPA from the resin. Moreover, elution with acetic acid is both safer and cheaper alternative to the methods used so far. This resin could be used for at least 3 cycles without any regeneration. Further studies are needed to determine the lifetime/number of cycles of the resin and the total product yield in order to determine the economy of this approach.

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Legends to figures

Fig. 1. Evaluation of binding of 3HPA (initial concentration of 71.54 ± 1.70 mM) to semicarbazide-functionalized resin for (A) 30 min and (B) 30 h in a batch mode at room temperature (22 °C).

Fig. 2. Adsorption equilibrium between the amount of 3HPA bound to the resin and the concentration of remaining 3HPA in solution (equilibrium concentration).

Fig. 3. Breakthrough curve of 3HPA on loading 25 ml of 3HPA solution (107.12 ± 1.27 mM [■] and 48.7 ± 0.53 mM [◆]) at a rate of 0.1 ml/min, and 3HPA solution (48.70 ± 0.53 mM) at a rate of 0.036 ml/min [▲] in a downward direction over a column packed with 0.5 g of semicarbazide-functionalized resin at 22 °C. Dotted lines indicate the initial 3HPA concentrations.

Fig. 4. (A) Effect of acetic acid concentration on the recovery of 3HPA from semicarbazide-functionalized resin, (B) total recovery of 3HPA (%) from the resin by 1st elution with deionised water (black bar) and 2nd elution with acetic acid (9 M) (white bar).

Fig. 5. Biotransformation of glycerol to 3HPA using *L. reuteri* with and without *in situ* removal using semicarbazide-functionalized resin (0, 0.5 and 1 g).

Scheme 1. (A) Reaction of 3HPA (1) with semicarbazide (2) to form 3HPA-semicarbazide complex (3) followed by dehydration to 3HPA-semicarbazone (4); (B) Preparation of semicarbazide functionalized resin. The compounds shown are aminomethyl polystyrene resin (5), 1,1'-carbonyldiimidazole (6), carbonyldiimidazole-amino methyl complex (7), *tert*-butyl carbazate (8); (C) Biotransformation of glycerol and complex formation of resulting 3HPA with semicarbazide resin. The compounds shown are 3HPA monomer (11), 3HPA hydrate (11a), and 3HPA dimer (11b) present as an equilibrium mixture in the aqueous solution, acrolein (11c), 1,3PDO (12), and 3HP (13) that are the normal end products from 3HPA, semicarbazide functionalized resin (10), and 3HPA-semicarbazide complex (14).

Table 1. Repeated adsorption-desorption of 3HPA using a batch equilibrium method with 0.2 g semicarbazide functionalized resin.

Cycle	3HPA concentration in loading solution (mM)			
1 st	48.70 ± 0.53	85.41 ± 3.41	48.70 ± 0.53	85.41 ± 3.41
2 nd	23.75 ± 1.06	46.44 ± 0.53	40.26 ± 0.53	74.56 ± 1.49
3 rd	14.63 ± 1.59	28.28 ± 0.21	36.42 ± 0.42	71.39 ± 1.91
Adsorption time	12 h	12 h	30 min	30 min
Adsorption (μmol)				
1 st	249.06 ±10.53	389.17 ±5.26	84.23 ±5.26	108.26 ±14.85
2 nd	89.23 ±5.13	177.78 ±3.10	37.53 ±0.94	30.91 ±4.05
3 rd	23.49 ±33.21	94.49 ±0.94	23.62 ±0.54	42.52 ±25.51
Total	361.77	661.45	145.38	181.70
1 st Elution with deionized water (μmol)				
1 st	15.79 ±0.40	28.35 ±0.94	20.65 ±0.40	34.83 ±2.43
2 nd	11.47 ±0.81	17.95 ±0.40	18.49 ±0.40	32.53 ±0.00
3 rd	8.37 ±0.54	13.23 ±0.40	17.55 ±0.27	33.75 ±0.54
Total	35.64	59.53	56.70	101.11
2 nd Elution with acetic acid (μmol)				
1 st	11.07 ±1.35	13.36 ±0.81	7.83 ±0.00	7.56 ±0.00
2 nd	6.21 ±0.00	7.69 ±0.67	6.07 ±0.13	6.21 ±0.00
3 rd	4.18 ±0.27	5.53 ±0.27	5.13 ±0.27	5.94 ±0.40
Total	20.25	26.59	19.03	19.71

Table 2. Batch biotransformation of glycerol to 3HPA using resting cells of *L. reuteri* with and without *in situ* 3HPA removal using semicarbazide-functionalized resin.

Resin	Consumed	Free	Bound	Total	Yield	Volumetric
(g)	glycerol	3HPA^(a)	3HPA	3HPA^(b)	(mol %)	Productivity
(mmol)	(mmol)	(mmol)	(mmol)	(mmol)		(mM/h)
0	0.46±0.037	0.19±0.07	--	0.19±0.07	42	7.01
0.5	0.56±0.02	0.01±0.00	0.29±0.01	0.3±0.00	53	10.81
1	0.74±0.04	0.01±0.00	0.43±0.06	0.44±0.05	59	16.01
^(a) Free 3HPA in solution						
^(b) Total 3HPA = Free 3HPA in solution + Bound 3HPA						











