Microbial Production of 3-Hydroxypropionaldehyde from Glycerol Bioconversion

C. Ulmer and A.-P. Zeng*
Institute of Bioprocess and Biosystems Engineering, Hamburg University of Technology, Denickestraße 15, D-21073 Hamburg, Germany
*aze@tu-harburg.de

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3-Hydroxypropionaldehyde (3-HPA) is of high industrial interest as a new platform intermediate from bioconversion of renewable materials because it can be converted into a number of large scale commodity chemicals. In this work we studied the production of 3-HPA from bioconversion of glycerol in a two-stage process. In the first stage active biomass is produced. The active biomass is then used for the production of 3-HPA in a second stage by biotransformation with the help of semicarbazide to trap 3-HPA from the culture. First we optimized the conditions for active biomass production for the biotransformation in the second step. By using a fed-batch process with proper feeding of semicarbazide and supplementary addition of active biomass we reached a final concentration of 54 g/l 3-HPA with a yield of 97 % (mol/mol). This represents the highest 3-HPA concentration and yield reported so far for the microbial production of 3-HPA from glycerol.

Key words:
3-Hydroxypropionaldehyde, glycerol, *Klebsiella pneumoniae,* biotransformation

Introduction

3-Hydroxypropionaldehyde (3-HPA) is of high industrial interest as a new platform intermediate because it can be relatively easily converted into a number of large scale commodity chemicals such as acrolein, 3-hydroxypropionic acid, acrylic acid, malonic acid and acrylamide. 3-HPA has also antimicrobial activity towards a wide range of pathogens and food spoilage organisms and is thus used as a food preservative and a therapeutic auxiliary agent in the food and pharmaceutical industries.[1–7] As a byproduct of biodiesel production glycerol nowadays is a very cheap and abundant substrate for the production of chemicals such as 3-HPA.

3-HPA is an intermediate of the reductive metabolism of glycerol by microorganisms, e.g. by species of *Enterobacteriacae* (Fig. 1) and its accumulation is quite toxic for growing organisms.[8–10]

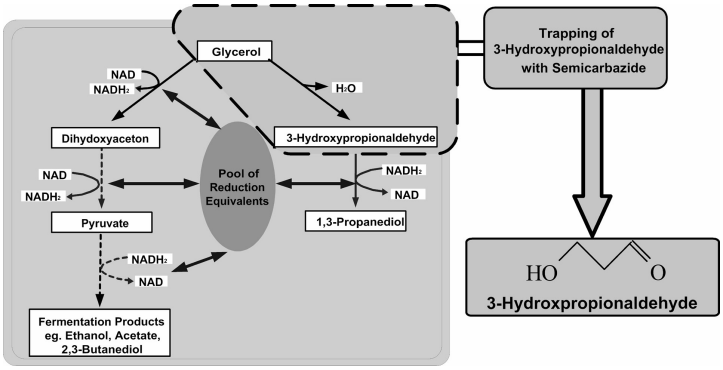


Fig. 1 – *Anaerobic metabolism of glycerol and trapping of 3-hydroxypropionaldehyde with semicarbazide*

Two major routes have been described to produce 3-HPA in significant amount and to avoid the toxicity. Both of them use a two-stage process in which active biomass is produced in the first stage and as second stage a resting cell process is applied.

The first route uses *Lactobacillus reuteri* as a production strain. 3-HPA can be produced heterofermentatively in the presence of glucose and glycerol.[10] However a significant amount of 3-HPA can only be reached by the application of a two-stage process.[11] Active biomass should be first obtained in a fermentation process which is then used in a biotransformation process using glycerol as single substrate.[11] The strain as well as the product reuterin (cyclic, dimeric 3-HPA) was patented.[12]

The second route uses *K. pneumoniae* as a production strain. First, active biomass is produced by glycerol fermentation and then used for the production of 3-HPA in a semicarbazide buffer (Fig. 1). Normally, glycerol will be converted to 1,3-propanediol by *K. pneumoniae* under anaerobic conditions. The maximal yield of 1,3-propanediol is restricted to 0.72 mol/mol because a part of the substrate is used to generate reduction equivalents which are needed to reduce 3-HPA to 1,3-propanediol. By using semicarbazide, 3-HPA can be trapped and its conversion to 1,3-propanediol is blocked. The theoretical yield for 3-HPA is 1 mol/mol glycerol. This method is based on the work of Abeles *et al.*[13] It was further developed[14–16] and patented by Slininger and Bothast.[17] An overview of the biomass- and glycerol concentrations used as well as the resulting final 3-HPA concentrations and yields of the described production methods are given in Table 1.

Table 1 – *Biotechnological production of 3-hydroxypropionaldehyde (3-HPA) reported in literature. Results from this study are also included.*

Strain	Biomass [g/l]	Initial Glycerol [g/l]	3-HPA produced [g/l]	$Y_{3\text{-HPA/Gly}}$ [mol/mol]	Source
L. reuteri	n. d.	27.6	11.1	0.64	Talarico *et al.*[10]
L. reuteri	30	18.4	12.6	0.85	Lüthi-Peng *et al.*[18]
L. reuteri	n. d.	18.4	12.6	0.85	Vollenweider *et al.*[11]
L. reuteri	$1.6 \cdot 10^{10}$ cfu*	36.8	17.4	0.62	Doleyres *et al.*[19]
K. pneumoniae	5	30.0	13.1	0.54	Slininger *et al.*[14]
K. pneumoniae	n. d.	30.0	16.9	0.70	Slininger and Bothast[15]
K. pneumoniae	14.5	70.0	46.0	0.82	Vancauwenberge *et al.*[16]
K. pneumoniae	10 + 4**	51 + 13**	54.0	0.97	This work

*cfu: **c**olony-**f**orming **u**nits

**added successively

n. d.: no data available

In this work we used *K. pneumoniae* DSM 2026 as a model organism to improve the production of 3-HPA by trapping with semicarbazide. The application of a fedbatch process led to a higher yield and a higher final concentration of 3-HPA. Commercially available glycerol was used in this study. In the future, it would be interesting to use crude glycerol directly from biodiesel plants. In previous studies of microbial production of 1,3-propanediol crude glycerol was shown to be applicable without difficulties.[20–24]

Materials and methods

3-HPA production was carried out using a two step process. The first step consists of the production of active biomass. The conditions for an optimal biomass production were first tested. In the second step glycerol is converted into 3-HPA by resting cells incubated in semicarbazide/phosphate buffer.

Strain

For the experiments *K. pneumoniae* DSM 2026 was used which was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSMZ).

Medium and culture conditions

The medium for active biomass production contained the necessary minerals,[25] 0.1 % yeast extract, 0.01 % (v/v) desmophen as antifoam agent and glycerol (Sigma-Aldrich >99 %) as indicated in each specific experiment. For active biomass production a 4-l fermenter (Set 4 V, Setric Genie Industriel, Toulouse, France) set-up with 2.5 l working volume was used. The bioreactor was connected to a real-time computer control system (Ubicon, ESD, Hannover, Germany) for online data acquisition. The cultivation conditions were as follows: temperature 37 °C, pH 7.0, and 400 rpm for agitation. The pH was regulated using alkaline solution (20 % NaOH).

The flow rate was adjusted at a value of 0.4 l l^{-1} min^{-1} using a mass flow meter (5850 TR, Brooks Instruments, Ede, Netherlands) and a mass flow controller (5876-2, Brooks Instruments).

The feeding solution for fed-batch fermentation of *K. pneumoniae* contained 85 % glycerol and 0.5 % yeast extract. Feeding of substrate was started after consumption of 10 g alkaline solution (20 % NaOH), normally about 4 h after inoculation. The feeding rate was coupled with alkaline solution consumption and the feeding factor was about 1.5–1.7 g/g (feed solution) per gram alkaline solution consumed.

Biotransformation

For the biotransformation process cells were harvested in the exponential phase of the biocatalysator production. Immediately after harvesting the fermentation broth was filled in 250 ml centrifugation bottles (Herolab, Wiesloch, Germany) and centrifuged in a cooling centrifuge (RC5C, Sorvall Instruments/Kendro, Düsseldorf, Germany) at 4 °C at 11000 rpm for 30 min. The supernatant was removed and the pellet resuspended with a buffer containing 40 g/l semicarbazide hydrochloride, 17.4 g/l K_2HPO_4 according to Vancauwenberge *et al*.[16] The pH was adjusted at pH 6 using NaOH. The cells were diluted with biotransformation buffer until the desired biomass concentra-

tion was reached. Subsequently glycerol was added and the biotransformation experiments were carried out at 30 °C.

Oxygen limited biotransformation experiments were performed using a 1–l fermenter (BCC, Göttingen, Germany) with pH and temperature control and with a working volume of 300 ml and anaerobic biotransformation experiments in 100 ml anaerobic flasks.

Analytical methods

Glycerol and lactate were assayed enzymatically using test kits from Roche-Biopharm (Darmstadt, Germany).

Fermentation products were measured gas-chromatographically with Chromosorb 101 as column material (Varian-Chrompack, Frankfurt, Germany) and a flame ionization detector (FID) as detecting method. Nitrogen was used as carrier gas and *n*-butanol as an internal standard.[26]

3-HPA was determined using a colorimetric method adapted from Circle *et al*.[27] 500 μl of properly diluted sample was mixed with 1 ml of 37 % HCl and 250 μl DL-tryptophan solution. The reaction time was 20 min at 40 °C. For measurement of samples and for preparing the standard curve triplicates were carried out. As standard for the 3-HPA measurement freshly distilled acrolein was used.

3-HPA for the semicarbazone formation was chemically synthesized from acrolein after a method reported by Hall and Stern[28] since 3-HPA is not commercially available. 3N sulphuric acid was used as catalyst. The solution was cooled down to 4 °C after the reaction (3 – 5 h) and neutralised by addition of calcium carbonate. Residual acrolein was removed after filtering by using a rotary evaporator.

Results and discussion

Examination of the mechanism

Until now the detailed mechanism of trapping 3-HPA with semicarbazide is not clear. Slininger and Bothast described the mechanism as a chemical entrapment of 3-HPA or as a reaction of 3-HPA with semicarbazide.[15]

It is known that aldehydes can form semicarbazones with semicarbazide in aqueous solutions under certain reaction conditions.[29] Semicarbazones are not soluble in water and a separation of the semicarbazones from the aqueous solution through precipitation is possible. Fig. 2 shows the possible reactions involved in the chemical trapping of 3-HPA and the formation of 3-hydroxysemicarbazone.

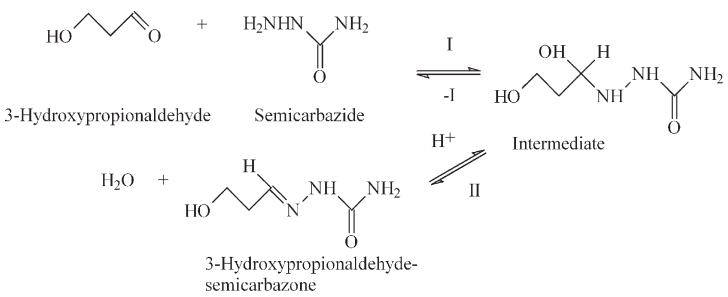


Fig. 2 – *Reactions involved in the trapping of 3-hydroxypropionaldehyde and formation of semicarbazone*

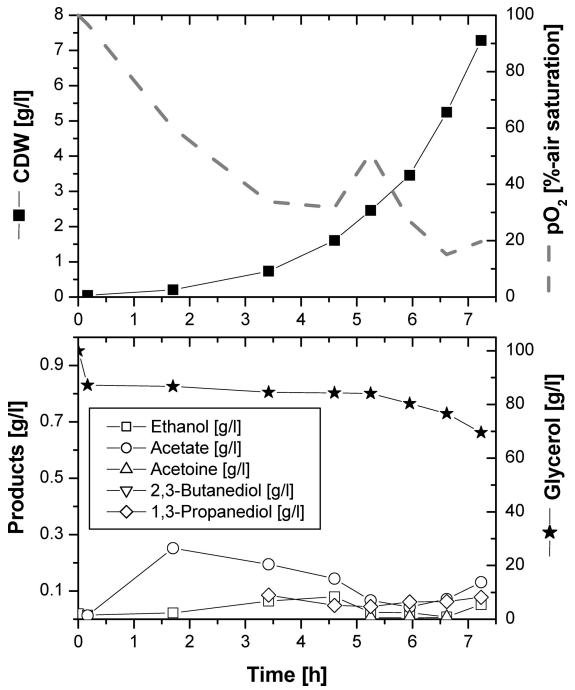
To examine this mechanism and to know whether 3-hydroxysemicarbazone is formed during the biotransformation process the formation of the 3-hydroxysemicarbazone was first determined at 30 °C using biotransformation buffer with a 4.25 fold higher semicarbazide concentration compared to the 3-HPA concentration. No 3-hydroxysemicarbazone was recorded by measurement of 3-HPA decrease within 16 h. When the experiment was repeated at 90 °C, 33 % of the 3-HPA was found in form of 3-hydroxysemicarbazone within 9 min. Because of this result and by the fact that semicarbazides and aldehydes react formation of an intermediate it can be proposed that the chemical entrapment of semicarbazide is based on the formation of the intermediate product of the semicarbazone reaction. This is further supported by the fact that at acidic to neutral pH values semicarbazone tends to hydrolyse and the dehydration of the intermediate is a slow reaction as indicated by Jencks[30] (Fig. 2).

Optimal conditions for active biomass production

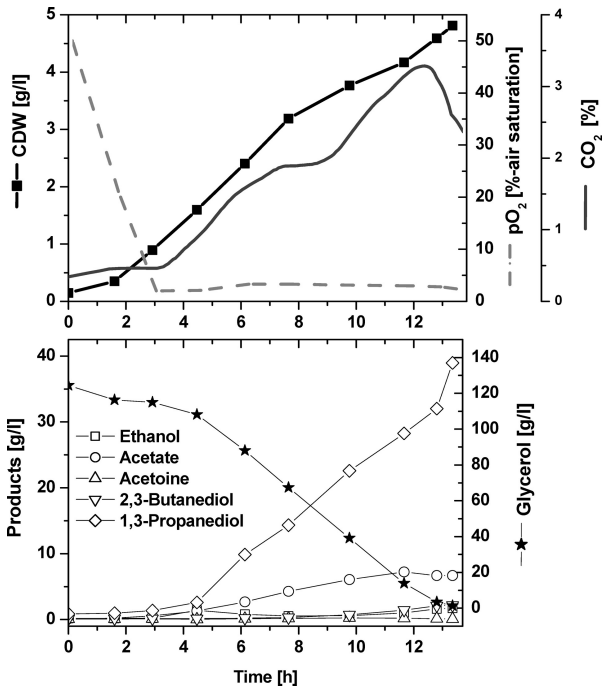
Slininger and Bothast stated that aerobic conditions are necessary to produce active biomass for 3-HPA production.[17] It is however known that the necessary enzyme for the 3-HPA formation, namely glycerol dehydratase the gene of which is a component of the *dha*-regulon,[31] normally is not expressed under strict aerobic conditions.[32]

In order to evaluate the influence of oxygen for the production of active biomass, batch fermentations were carried out under anaerobic, aerobic and oxygen limiting conditions. Biotransformation experiments were also done under anaerobic and oxygen limiting conditions to examine the efficiency of glycerol conversion to 3-HPA by biomass generated under different conditions.

Aerobic culture. In order to maintain strict aerobic conditions in the culture dissolved oxygen concentration (pO_2) was set to be controlled above 30 % of air saturation by feeding molecular oxygen to the aeration gas at a high agitation speed (1000



F i g . 3 – *Bioconversion of glycerol and generation of active biomass under aerobic conditions*



F i g . 4 – *Bioconversion of glycerol and generation of active biomass under oxygen limiting conditions*

rpm) of the bioreactor. 100 g/l of initial glycerol concentration was used in this batch fermentation. These conditions could only be kept within the first 6h of the cultivation (Fig. 3). After this period, the pO_2 value decreased below 30 %. The cultivation was terminated shortly after that. A maximal dry cell weight (DCW) of 7.3 g/l with a maximum growth rate of 0.84 h^{-1} was reached and 30.5 g of glycerol was used. This growth rate is significantly higher than the maximum growth rate (0.71 h^{-1}) reported for *K. pneumoniae*[33] grown anaerobically on glycerol at the same pH value.

The biomass was harvested and used for the biotransformation experiments both under oxygen limiting and anaerobic conditions. No 3-HPA was detected under aerobic conditions and only 0.2 g/l under oxygen limiting conditions in the following biotransformation process, revealing that no active biomass was produced under strict aerobic conditions.

Culture with oxygen limitation. To examine oxygen limiting conditions batch fermentation with 125 g/l of initial glycerol concentration and aeration with a gas mixture of 50 % N_2 to 50 % air at a rate of 0.4 l l^{-1} min^{-1} was carried out (Fig. 4). After 3 h oxygen limitation was reached and the *dha*-regulon was induced as evidenced by an increased 1,3-propanediol production. After 13 h a maximal dry cell weight of 4.8 g/l with a maximal growth rate of 0.62 h^{-1} was reached and a 1,3-propandiol concentration of 39 g/l was obtained. The major by-product in the culture was acetate.

The biomass from this culture was tested in the biotransformation step under both oxygen limiting condition and anaerobic conditions. With 10 g/l biomass a maximal 3-HPA concentration of 35 g/l was obtained within 3.5 hours of biotransformation under oxygen limiting conditions, resulting in a yield of 72 % (mol/mol) and volumetric productivity of 10 g l^{-1} h^{-1}. With the same amount of biomass under anaerobic conditions a maximal 3-HPA concentration of 29.5 g/l was obtained with a somewhat lower yield (62 % mol/mol) and a lower productivity (7 g l^{-1} h^{-1}). This indicates that for the biotransformation step oxygen limiting conditions are more favorable than anaerobic conditions.

Anaerobic culture. Glycerol conversion to 1,3-propanediol is normally carried out under anaerobic conditions. We evaluated if anaerobic conditions are also appropriate for the production of active biomass for biotransformation of glycerol to 3-HPA and how the results can be compared with those from biomass generated under oxygen limiting conditions. To this end, 110 g/l initial glycerol concentration was taken and in order to get anaerobic conditions, N_2 was dispersed at a rate of 0.4 l l^{-1} min^{-1}. A maximal cell dry mass concentration of 4.2 g/l with a maximal growth rate of 0.48 h^{-1} was reached (data not shown). In this fermentation 117 g of glycerol were consumed and 39 g/l 1,3-propanediol were reached, resulting in a yield of 49 % (mol/mol).

The biomass (in a concentration of 10 g/l) from this culture was also tested in the biotransformation step under both oxygen limiting conditions and anaerobic conditions. Under oxygen limiting conditions a maximal 3-HPA concentration of 27 g/l was obtained within 3.8 h with a yield of 76 % (mol/mol) and volumetric productivity of 7.1 g l^{-1} h^{-1}. Under anaerobic conditions a maximal 3-HPA concentration of 22.1 g/l was obtained with a somewhat lower yield (70 % mol/mol) and a lower productivity (5.4 g l^{-1} h^{-1}). This indicates that for the biotransformation step oxygen limiting conditions are more favorable than anaerobic conditions.

Comparing all the conditions for the production of biomass as well as for the biotransformation step as summarized in Table 2, it is obvious that oxygen limiting conditions are favorable for both the production of active biomass and the biotransformation.

Table 2 – *Comparison of conditions for biocatalysator production and biotransformation of glycerol to 3-hydroxypropionaldehyde*

Conditions for biomass production	Conditions for biotransformation	$C_{3\text{-HPA}}$ [g/l]	$Q_{3\text{-HPA}}$ [g/lh]	$Y_{HPA/Gly}$ [mol/mol]
Oxygen limiting	Oxygen limiting	35.1	10.0	0.72
–	Anaerobic	29.5	6.9	0.62
Anaerobic	Oxygen limiting	27.1	7.1	0.76
–	Anaerobic	22.1	5.4	0.70

To explain the above results it is worth to mention that glycerol dehydratase (GDHt), the enzyme responsible for the conversion of glycerol into 3-HPA, is vitamin B12 dependent. Previously it was reported for the microorganism *Rhodopseudomonas gelatinosa* that vitamin B_{12} is optimally produced under oxygen limiting conditions.[34] This may also be the case for *K. pneumoniae*. Furthermore, GDHt normally undergoes a mechanism-based inactivation by the substrate glycerol during catalysis. A reactivation factor mediates an ATP-dependent exchange of the enzyme-bound, adenine-lacking cobalamin for free, adenine-containing cobalamin.[35–39] This reactivation process may be also favored by oxygen limiting conditions from a bioenergetic point of view since the ATP generation under oxygen limiting conditions is higher than under anaerobic conditions.

Optimization of biotransformation in fed-batch process

In this part of the work, it was examined if the yield and final concentration of 3-HPA in the biotransformation step can be further improved by a fed-batch process feeding semicarbazide to the biotransformation process.

The biomass for the biotransformation was also first obtained from a fed-batch culture under oxygen limiting conditions. In order to reach oxygen limiting conditions fast an aeration with a gas mixture of 80 % N_2 and 20 % air was applied. Yeast extract was fed linearly within the first 4 hours to accelerate biomass production. After 8 h the active biomass from exponential growth phase was harvested and used for the subsequent biotransformation experiment.

We first examined if feeding of semicarbazide can lead to an improvement in 3-HPA yield. A supplementary addition of new biomass should indicate if an increase of 3-HPA concentration is possible under a high semicarbazide concentration.

In the first phase (the first 4 h) of the biotransformation process semicarbazide was fed continuously from 40 g/l to a final concentration of 92 g/l (Fig. 5). A very high yield of 98 % (mol/mol) was achieved, but with only a modest concentration of 20 g/l 3-HPA. The formation of byproducts was also strongly reduced. This is probably due to the strong inhibition or toxicity of semicarbazide at such a high concentration. In the second phase of this experiment the semicarbazide concentration was kept constant at 92 g/l and new active biomass

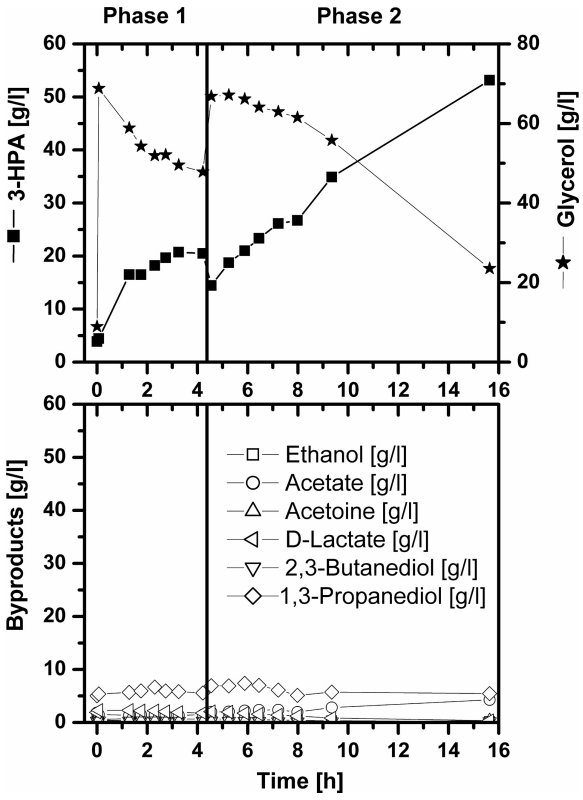


Fig. 5 – *Optimization of the biotransformation step. Phase 1: Feeding of semicarbazide (40 g/l – 92 g/l), CDW=10 g/l; Phase 2: Addition of new active biomass (4 g/l)*

and substrate was added to achieve a final biomass concentration of 14 g/l and a glycerol concentration of 67 g/l. Here within further 11 h a final concentration of 54 g/l 3-HPA was obtained. The yield was also very high (97 % mol/mol). The overall productivity was 3.5 g l^{-1} h^{-1}. In a similar experiment with lower semicarbazide concentrations in the first stage (40 g/l) and second stage (62 g/l) the production of byproducts was much higher (data not shown), resulting in a lower overall yield (83 % mol/mol) and 3-HPA concentration (37 g/l).

It can be concluded that the higher the semicarbazide concentration, the higher the achieved yield. This is obviously caused by the fact that semicarbazide is quite toxic to the cells as well as caused by the mechanism of this biotransformation which at higher concentrations will totally stop the metabolism of the bacterium. This can lead to a reduced volumetric productivity. An optimum between high concentration, product formation rate, cell dry weight concentration and yield has to be chosen. Feeding of semicarbazide and addition of new biomass is a proper strategy to solve this paradox. The final 3-HPA concentration (54 g/l) and the yield (97 % mol/mol) achieved in the fed-batch process described above represent in fact the highest 3-HPA concentration and yield reported so far for the microbial production of 3-HPA from glycerol (Table 1).

Conclusion

For the microbial production of 3-hydroxypropionaldehyde from glycerol by *K. pneumoniae* in a two-step process oxygen-limiting conditions are favourable for both the production of active biomass and the biotransformation step. By applying a fed-batch process with high semicarbazide concentration and successive addition of active biomass a 3-HPA yield (97 % mol/mol) near the theoretical value can be achieved. A final 3-HPA concentration as high as 54 g/l was achieved in this study which represents the highest 3-HPA concentration reported so far.

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