Production of 3-hydroxypropionaldehyde with immobilised *Lactobacillus reuteri* cells

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Introduction

3-hydroxypropionaldehyde (3-HPA) forms an equilibrium with its hydrate and its dimer (reuterin) (Hall 1950, Dobrogosz 1989). It inhibits bacteria, moulds, yeasts and protozoa including human pathogenic and food spoilage organisms (Dobrogosz 1989, El-Ziney 1998). Furthermore, 3-HPA is a precursor for 1,3-PDO, which has several applications in the chemical industry. However, it is not commercially available (Vollenweider 2004).

A promising approach is the biotechnological production from glycerol with bacteria converting glycerol to 3-HPA by a cobamide-dependent glycerol dehydratase, it can be further reduced to 1,3-PDO by a NADH-linked dehydrogenase as recently reviewed by Vollenweider (2004). For the biotechnological production of 3-HPA in high concentrations a 3-HPA accumulating bacterium, *Lactobacillus reuteri* (*Lb. reuteri*) is prevented from reducing 3-HPA to 1,3-PDO by separating biomass production from glycerol conversion in a two step process (Lüthi-Peng 2002). In free cell (FC) culture, 3-HPA production was shown to depend on the initial biomass concentration, temperature, glucose availability and the glycerol concentration (Lüthi-Peng 2002, Doleyres 2005). The process has several limitations which may be solved with immobilised cells (IC): required centrifugation steps for medium change, complete loss in viability and the little applicability for continuous production and *in-situ* product removal. Apart, other advantages of IC have been reported such as high cell densities and productivities, low susceptibility to contamination (Lacroix 2005). To our knowledge, no publication has reported the production of 3-HPA with IC until now.

The accumulation of excreted 3-HPA during production, leads not only to a reduction of unwanted microorganisms but also inhibits and kills producing cells which limits final 3-HPA concentration (Doleyres 2005). A reversible *in-situ* binding of 3-HPA to selected complexing agents during glycerol conversion could decrease the toxicity, increase productivity and simplify purification.

The aim was to study glycerol conversion with IC. Furthermore, an *in-situ* product removal process was developed to simplify the 3-HPA purification by selective binding of the 3-HPA to ligands.

Material and methods

Strain and medium

The stock culture of *Lb. reuteri* (SD 2112), obtained from Biogaia (Stockholm, Sweden), was kept at -80°C in a solution containing 10 % (w/v) glycerol and 6 % (w/v) skim milk powder. The inoculum was prepared in MRS broth (Biolife) containing 20 mM glycerol from 1 % frozen stock culture and incubated for 16 h at 37°C in a still culture. All media and solutions were prepared with bidistilled water and autoclaved at 121°C for 20 min unless indicated otherwise.

Immobilisation and colonisation

The immobilisation procedure was adapted from a method by Cinquin (2004). Briefly, *Lb. reuteri* cells are immobilised in a gellan/xanthan gum polysaccharide matrix. The autoclaved gel was

cooled to 45°C, inoculated with an overnight culture (2 % v/v), poured into sterile oil (45°C), cooled down to form beads and hardened by incubating in cold (4°C) 0.1 M $CaCl_2$ solution for 30 min. Beads with diameters in the range of 1.0–2.0 mm range were selected by wet sieving.

Bead colonisations were carried out in MRS broth containing 20 mM glycerol either by a 6-h batch and a 16-h continuous ($D = 0.5\ h^{-1}$) or by a 16-h batch and a 24-h continuous ($D = 1.5\ h^{-1}$) fermentation with or without added 40 g/l glucose. Other parameters were pH 5.5 controlled with 5 M NaOH, 37°C, inoculum of 30 % (v/v) beads in the reactor and headspace flushed with N_2 (4 l/h at 20°C and 1atm). After colonisation, beads were washed with 0.1 M $CaCl_2$ solution and used for 3-HPA production.

3-HPA production

For all conversions, a 500 ml reactor (working volume 250 ml) containing 25 ml beads (10 % v/v) and 225 ml glycerol solution (400 mM) were used. Headspace was flushed with N_2 (4 l/h) to maintain anaerobic conditions. For the *in-situ* 3-HPA removal production, two 150 ml columns were packed with a scavenger. During conversion, the first column was replaced after 1 h, the second was then kept until the end.

Metabolite and bacteriological analyses

Samples (3 ml) were centrifuged at 14000*g* for 5 min and the supernatant was filtrated through a 0.45 µm nylon HPLC filter. 3-HPA, glycerol, 1,3-PDO were determined by HPLC (10 mM H_2SO_4, Animex HPX-87H column, RI) and quantified by external standards of commercially pure substances, except for 3-HPA, which was purified using a silica gel 60 column which was eluted with acetone:ethyl acetate (2:1) (Vollenweider 2003).

Viable cell count determinations in beads were performed by dissolving 0.5 g accurately weighed beads in 20 ml 1 % EDTA solution (pH 7), followed by a serial dilution series and incubated anaerobically on MRS agar at 37°C for 48 h (Cinquin 2004). To detect contamination, a sample was observed under the microscope and plated out on LB agar (37°C, 72 h).

Results and Discussion

Effect on the colonisation method on cell concentration in beads

Three different colonisation methods were tested in this study. Cell counts in beads were in the range from $6.2 \cdot 10^{10}$ CFU/g to $1.8 \cdot 10^{11}$ CFU/g with the highest concentration achieved by the 16-h batch and 24-h continuous fermentation in MRS without glucose supplementation.

Influence of the initial biomass concentration on the production of 3-HPA

The effect of different initial biomass concentrations in beads was studied for high 3-HPA production (Fig. 1) during glycerol bioconversion carried out at 20°C in 400 mM glycerol, cell counts ranged from $6.2 \cdot 10^{10}$ CFU/g to $1.8 \cdot 10^{11}$ CFU/g. As expected, the higher the cell concentration, the higher the 3-HPA production from 57 mM to 183 mM 3-HPA respectively. A linear correlation (R^2=0.94) was measured between initial cell concentration and 3-HPA production after 1 h. Conversion started with no lag time and the highest productivity was recorded in the first 5 min of the conversion. No loss in viability was detected after 1 h of incubation.

These data were compared with published data for FC containing an overall cell density of $1.6 \cdot 10^{10}$ CFU/ml in the reactor (Doleyres 2005). In comparison to FC, 3-HPA production with IC was the same but survival of the producing *Lb. reuteri* strain was improved. Concretely, after 1 h of incubation, reduction of viable cells in beads was already two decades whereas in the production with FC no viable cells were detectable.

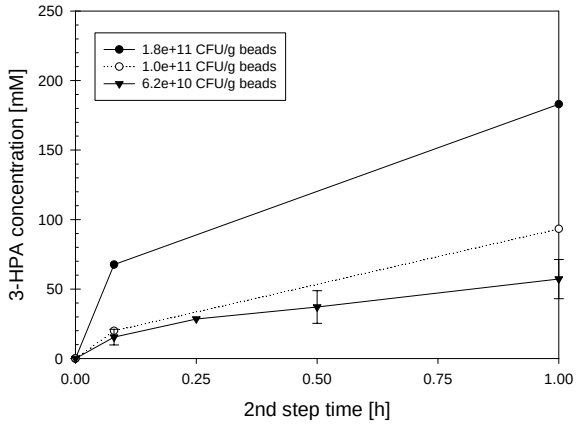

Fig. 1. Influence of the initial cell concentration on glycerol conversion in 400 mM glycerol solution at 20°C using 10 % (v/v) colonised beads. *Error bars* standard deviations of 3 repetitions.

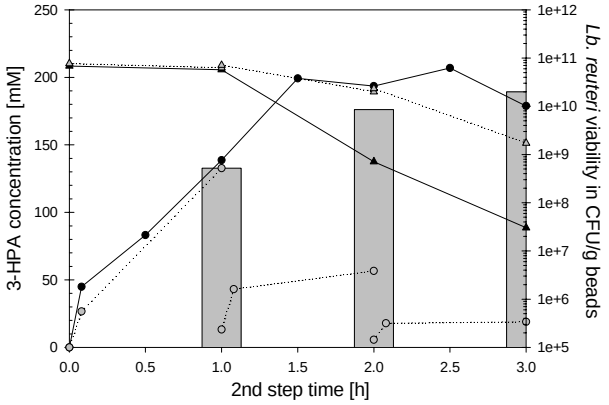

Fig. 2. 3-HPA production (●,●) and *Lb. reuteri* viability (▲,▲) during three successive 1-h (●,▲) or one 3-h (●,▲) incubations at 30°C in 400 mM glycerol. Cumulated 3-HPA production (■) of 3x1 h incubations(●).

Repeated glycerol conversions with immobilised cells

Three successive 1-h incubations in 400 mM glycerol were carried out at 30°C for an initial biomass concentration in the first incubation of $7.3 \pm 0.6 \cdot 10^{10}$ CFU/g. 3-HPA production decreased after each incubation cycle, with final 3-HPA concentrations of 133, 57, and 19 mM corresponding to cumulative productions of 133, 176, and 189 mM determined at the end every cycle (Fig. 2). In comparison with the corresponding 3-h conversion (Fig. 2), in successive batches 3-HPA accumulation was the same but the viability of *Lb. reuteri* cells were improved due to lower 3-HPA exposure during conversion. However, to take into account the higher glycerol input and still measurable decrease of viability, a 3-h conversion seems better suited for 3-HPA accumulation.

Glycerol conversion with *in-situ* product removal

A new process for simplified *in-situ* recovery and increased production was tested for 3-HPA production. A 3-h incubation was carried out at 20°C in 400 mM glycerol solution, which was pumped continuously ($D=1\ h^{-1}$) through a column containing an aldehyde scavenger to bind 3-HPA and back to the fermenter. Beads colonised with $1.2 \cdot 10^{11}$ CFU/g were used. Glycerol consumption, 3-HPA and 1,3-PDO concentrations in the fermenter are presented in Fig. 3. As expected, 3-HPA, which was retained in the column, and 1,3-PDO concentrations were low (around 4 mM), where glycerol decreased from 400 mM to 165 mM. Dilution with water adjusted (*dashed line*), glycerol consumption was 114 mM which was all presumably converted to 3-HPA fitting in the data shown in Fig. 1. Viable cell counts in beads remained very high during the incubation period (end cell counts $6.2 \cdot 10^{10}$ CFU/g). This reduction is probably due to a lack of glucose and not due to the toxicity of 3-HPA. Release of 3-HPA from the column was successful.

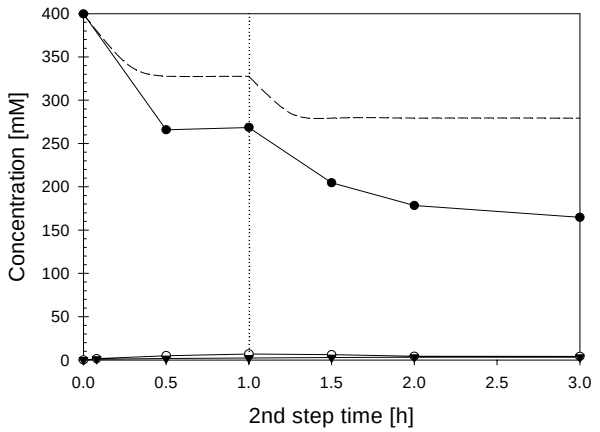

Fig. 3. Glycerol consumption (●), 3-HPA (○) and 1,3-PDO (▾) accumulation during glycerol conversion with $1.2 \cdot 10^{11}$ CFU/g *Lb. reuteri* cells at 20°C. Continuous product removal by a scavenger. *Dashed line* expected decline of glycerol due to dilution by water from the column.

Conclusions

In this study it was shown that IC can be used for the bioconversion of glycerol to 3-HPA with advantages over FC conversions concerning viability, handling and *in-situ* removal, although the production was comparable to FC. A linear regression between initial cell density and 3-HPA production was observed urging to highest cell density which did not increase with glucose addition to MRS. The *in-situ* product removal process allowed to retain 3-HPA in the column and keep a low concentration of toxic 3-HPA in the reactor. Interestingly, production stopped after 3 h, even though a high cell count $6.2 \cdot 10^{10}$ CFU/g was still in beads. An explanation for this effect could be the lack of glucose which reduced all activity.

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