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Author(s):
Doleyres, Y.; Beck, P.; Vollenweider, S.; Lacroix, C.

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Y. Doleyres · P. Beck · S. Vollenweider · C. Lacroix

Production of 3-hydroxypropionaldehyde using a two-step process with *Lactobacillus reuteri*

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Abstract 3-Hydroxypropionaldehyde (3-HPA) produced by *Lactobacillus reuteri* is a broad-spectrum antimicrobial substance of glycerol conversion. The aim of the present work was to optimize 3-HPA production by *Lb. reuteri* ATCC 53608 using a two-step process. The first step was the production of *Lb. reuteri* cells in optimal conditions. Cells were then harvested by centrifugation and suspended in glycerol solution, which the resting cells bioconverted to 3-HPA. The effect of biomass concentration, temperature, glycerol concentration, anaerobic/micro-aerophilic conditions, and incubation time was studied for high 3-HPA production. 3-HPA accumulation was limited by the death of cells in contact with high concentrations of 3-HPA. However, a very high 3-HPA concentration of 235±3 mM was obtained after 45 min of incubation at 30°C in 400 mM glycerol for an initial free-cell concentration of 1.6±0.3×10^{10} viable cells/ml. A high viability was maintained at low temperatures in the range 5–15°C, but with a slightly lower yield of 3-HPA at 5°C compared with higher temperatures, up to 37°C. Successive 1-h incubations of *Lb. reuteri* cells in 200 mM glycerol at 15°C to tentatively reuse the cells resulted in decreasing 3-HPA concentrations at the end of each cycle, with two successful production cycles yielding high 3-HPA concentrations of 147±1 mM and 128±2 mM.

Introduction

Lactobacillus reuteri is a bacterial strain known to inhabit the gastrointestinal tract of humans, swine, poultry, and other animals (Casas and Dobrogosz 2000). In glycerol-containing media, it has the ability to excrete high amounts of 3-hydroxypropionaldehyde (3-HPA), a potent bacterial inhibitor (Axelsson et al. 1989) which in aqueous solution undergoes a reversible dimerization and hydration, forming an equilibrium mixture of 3-HPA, HPA hydrate, and HPA dimer, also called reuterin (Vollenweider et al. 2003). Until now, five other genera of bacteria that are able to transform glycerol into 3-HPA have been identified (*Bacillus*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*) and from all of these *Lb. reuteri* produced the highest concentration of 3-HPA in pure glycerol solution (Vollenweider and Lacroix 2004).

Reuterin has antimicrobial activity towards a wide range of pathogens and food spoilage organisms, including both Gram-positive and Gram-negative bacteria, yeasts, moulds, and protozoa, which makes it attractive for use as a food preservative or as a therapeutic auxiliary agent, as recently reviewed by Vollenweider and Lacroix (2004). 3-HPA is also a precursor in the production of several industrial chemicals, such as 1,3-propanediol (1,3-PDO).

Under anaerobic conditions, glycerol is converted by *Lb. reuteri* into 3-HPA by a cobamide-dependent glycerol dehydratase (Smiley and Sobolov 1962). Unfortunately, 3-HPA is normally an intracellular intermediate that does not accumulate but is reduced to 1,3-PDO by a NADH-linked dehydrogenase (1,3-PDL:NAD+ oxidoreductase). The regulation of this pathway is dependent on the availability of fermentable carbohydrates, in particular glucose (Lüthi-Peng et al. 2002a). In fact, the presence of glycerol and its transformation into 1,3-PDO enables the cells to recover the NAD+ used during glycolysis. Under conditions of unlimited glucose, 1,3-PDO is formed, but the accumulation of 3-HPA by *Lb. reuteri* can still occur with a low yield if the molar ratio of glucose to glycerol in the growth medium is no greater than 0.33 (El-Ziney et al. 1998; Lüthi-Peng et al. 2002a).

A two-step process was thus proposed for high 3-HPA accumulation (Lüthi-Peng et al. 2002a, b). *Lb. reuteri* cells were first propagated in optimal conditions for cell growth and 3-HPA was subsequently produced by washed cells

Y. Doleyres · P. Beck · S. Vollenweider · C. Lacroix (✉)
Laboratory of Food Biotechnology,
Institute of Food Science and Nutrition,
Swiss Federal Institute of Technology,
ETH Zentrum, Schmelzbergstrasse 9,
Zurich, 8092, Switzerland
e-mail: christophe.lacroix@ilw.agrl.ethz.ch
Tel.: +41-1-6324867
Fax: +41-1-6321403

incubated in a pure aqueous glycerol solution. Using this method, 170 mM 3-HPA (12.6 g/l) was produced with a dry cell weight of 30 g/l, representing 85% of the glycerol supplied (200 mM in H_2O), within 2 h of incubation (Lüthi-Peng et al. 2002b). In the same study, 3-HPA accumulation by *Lb. reuteri* was investigated in water, milk, and MRS medium containing glycerol and was shown to be greatly affected by temperature, pH, cell age, biomass concentration, and the components of the medium. However, there are two major advantages in producing 3-HPA from a pure solution of glycerol compared with complex media such as milk or MRS medium supplemented with glycerol: first, no efficient 3-HPA purification from a complex growth medium has yet been reported, and second, the production of undesired side-products can be suppressed.

The aim of this study was to increase 3-HPA production by optimizing the parameters of the two-step fermentation process with *Lb. reuteri*. Therefore, the effect of different parameters (biomass concentration, temperature, glycerol concentration, incubation time) was studied to achieve maximal 3-HPA accumulation during glycerol bioconversion, since previous studies using a two-step process had been carried out under unoptimized conditions, particularly with very low biomass concentrations (Lüthi-Peng et al. 2002a, b). Additionally, the effect of anaerobic conditions during cell growth and glycerol bioconversion was compared to micro-aerophilic conditions. Cell viability and 3-HPA concentration were measured over time during glycerol bioconversion to 3-HPA to study the toxicity of 3-HPA towards the producing strain itself, which was reported to limit the accumulation of 3-HPA (Vollenweider and Lacroix 2004). Finally, the feasibility of reusing cells of *Lb. reuteri* was investigated during successive cell incubations in glycerol.

Materials and methods

Strain and medium

The stock culture of *Lb. reuteri* ATCC 53608 was kept frozen at −80°C in a solution containing 6% skim milk powder and 10% glycerol. For inoculum preparation, 1% of the frozen *Lb. reuteri* was propagated in MRS medium (de Man et al. 1960; Biolife, Milan, Italy) at 37°C for 15 h.

Biomass production

3-HPA production was carried out using a two-step process, which consisted of the production of *Lb. reuteri* cells under optimal conditions for cell growth (first step), followed by the biotransformation of glycerol to 3-HPA by resting cells incubated in aqueous glycerol solution (second step; Lüthi-Peng et al. 2002a, b).

The first step consisted in a 15-h batch culture in MRS medium supplemented with 20 mM glycerol (MRS+; Axelsson et al. 1989) inoculated with an overnight culture of *Lb. reuteri* (1%), using a Sixfors bioreactor (500 ml useful volume; Infors, Bottmingen, Switzerland), with mixing at 150 rpm. The temperature was set at 37°C, and the pH was held at 5.5 using 5 M NaOH for optimal cell growth. The initial pH of MRS+ medium was 6.2 and pH control started when the pH reached the defined set-point. Unless otherwise indicated, nitrogen (N_2) was injected into the bioreactor headspace to keep anaerobic conditions. Cells were then harvested by centrifugation at 1,500 *g* for 10 min at 20°C, washed with potassium phosphate buffer (0.1 M) at pH 7.0, centrifuged again under the same conditions, and immediately tested for 3-HPA production.

3-HPA production from glycerol

Cell pellets from the previous step were suspended in distilled water containing glycerol for its bioconversion to 3-HPA. The effects of time (0, 30, 45, 60, 90, 120 min), temperature (5, 15, 23, 30, 37°C), initial biomass concentration [from 7.0×10^8 colony-forming units (cfu)/ml to 4.5×10^{10} cfu/ml], and glycerol concentration (200, 250, 300, 400 mM) on the production of 3-HPA were studied independently. The effect of N_2 addition during the first and second steps of the two-step process was also studied. To test the effect of N_2 during the first biomass production step, no N_2 was used during the second bioconversion step, whereas N_2 was used for both cell growth and conversion when testing the effect of N_2 during the second step.

To test the reuse of *Lb. reuteri* cells after 3-HPA production, an initial short incubation period (1 h) of cells in 200 mM glycerol at 15°C was carried out. These conditions were chosen from experimental data to allow high glycerol bioconversion and high cell viability. Cells were then harvested by centrifugation at 3,000 *g* for 10 min at 15°C, washed with potassium phosphate buffer, centrifuged again, and resuspended in glycerol solution for 3-HPA production under the same conditions. This procedure was repeated twice.

Samples for viable cell enumeration and 3-HPA quantification were taken periodically during 2-h experiments, or at the beginning and end of each production period during repeated incubations. Dilution and plating of samples for viable cell enumeration were carried out immediately after sampling. Samples for 3-HPA quantification were centrifuged at 15,000 *g* for 5 min; and the supernatant was sterile-filtered (0.22 μm) and stored at 5°C until measurement. Reported data are means for duplicate experiments and analyses, except for the effect of initial biomass concentration, which reports only duplicate analyses.

Viable cell enumeration

Viable cell counts were determined by plating diluted (peptonized water) or non-diluted samples on MRS agar plates (detection limit of 1 cfu/ml) followed by 48 h of aerobic incubation of the plates at 37°C. Reported data are means for duplicate analyses.

3-HPA quantification

3-HPA quantification was carried out using a colorimetric method adapted from Circle et al. (1945). To obtain standard curves, 0–6 μmol of freshly distilled acrolein (Fluka, Buchs, Switzerland) was added to 6 ml of distilled water. Then, 4.5 ml of DL-tryptophan (Fluka) solution (0.01 M solution in 0.05 M HCl, stabilized with a few drops of toluene) and 18 ml of 37% HCl were added immediately. For 3-HPA quantification, a 1-ml sample was mixed with 0.75 ml of DL-tryptophan solution and 3 ml of HCl 37%. Mixtures containing samples and standards were incubated for 20 min in a water bath at 37°C and the optical density was measured at 560 nm (OD_{560}). 3-HPA samples were diluted with distilled water before mixing with reagents to ensure a final OD_{560} <1. This method was shown to allow a precise quantification of 3-HPA using acrolein as a standard (Lüthi-Peng et al. 2002a, b). The same tryptophan solution was used for the standard curves and all 3-HPA quantifications and reported data are means for duplicate analyses.

Statistical analysis

ANOVA was performed using the general linear model of SPSS ver. 11.0 (SPSS, Chicago, Ill.). Significant differences among treatment means were tested using the LSD multiple comparison test, with a level of significance of 0.05.

Results

Growth of *Lb. reuteri*

Mean final concentrations of *Lb. reuteri* after 15 h of pH-controlled batch culture at 37°C in MRS+ with and without N_2 addition in the headspace of the bioreactor were 1.6±0.4×10^9 cfu/ml and 1.7±0.3×10^9 cfu/ml, respectively. A fermentation time of 15 h was chosen for 3-HPA production to harvest cells in the stationary growth phase (data not shown).

Effects of biomass concentration during glycerol bioconversion on 3-HPA production

The effect of an initial biomass concentration ranging from 7.0×10^8 cfu/ml to 4.5×10^{10} cfu/ml on 3-HPA production during glycerol bioconversion carried out at 30°C in 200 mM glycerol was studied for high 3-HPA production. A 30-min delay with almost no 3-HPA production was observed for all biomass concentrations tested (Fig. 1a). Increasing the initial biomass concentration from 7.0×10^8 cfu/ml to 1.6×10^{10} cfu/ml led to an increased 3-HPA production (from 26 mM to 160 mM) and a decreased incubation time for maximal 3-HPA production (from 2 h to 45 min). However, the highest initial biomass concentrations of 2.5×10^{10} cfu/ml and 4.5×10^{10} cfu/ml surprisingly resulted in low 3-HPA concentrations of 134 mM and 64 mM, respectively, determined after 45 min of incubation. For 1.6×10^{10} cfu/ml and higher initial biomass concentrations, 3-HPA was produced very rapidly, to reach a peak after 45 min and then the concentration decreased slightly, whereas the highest 3-HPA productions with lower biomass concentrations were obtained at the end of the 2-h experiments. Cell viability during 3-HPA production dropped faster for increasing biomass concentrations, even though low 3-HPA productions were obtained at the highest biomass concentrations tested (Fig. 1b). No viable cells were thus detected by plate counts after 1 h for 4.5×10^{10} cfu/ml and 2.5×10^{10} cfu/ml, 1.5 h for 1.6×10^{10} cfu/ml, and 2 h for 8.5×10^9 cfu/ml; and very few viable cells (respectively 4.8×10^6 cfu/ml and 1.8×10^4 cfu/ml) were detected after 2 h for 2.3×10^9 cfu/ml and 5.3×10^9 cfu/ml (initial biomass concentrations). Finally, no cell death was observed during the 2-h incubation for the lowest cell concentration tested (7×10^8 cfu/ml).

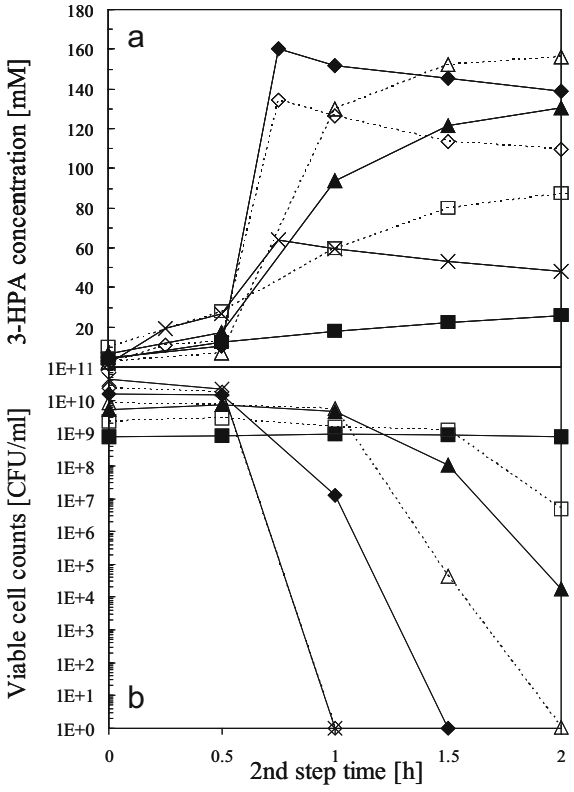

Fig. 1 Effects of initial biomass concentration on: **a** 3-HPA production and **b** *Lb. reuteri* viability during the glycerol bioconversion step. The temperature was 30°C and glycerol concentration 200 mM. *Filled squares* 7.0×10^8 cfu/ml, *open squares* 2.3×10^9 cfu/ml, *filled triangles* 5.3×10^9 cfu/ml, *open triangles* 8.5×10^9 cfu/ml, *filled diamonds* 1.6×10^{10} cfu/ml, *open diamonds* 2.5×10^{10} cfu/ml, *crosses* 4.5×10^{10} cfu/ml

Effects of N_2 addition during cell growth on 3-HPA production

The effects of N_2 addition during cell growth on the following 3-HPA production step were tested for three different biomass concentrations in the second step, with cells incubated for 2 h at 30°C in 200 mM glycerol. 3-HPA productions by cells grown with N_2 were slightly but significantly ($P<0.05$) higher than without N_2 (Fig. 2a). For an initial cell concentration of $1.7\pm0.3\times10^{10}$ cfu/ml, maximal 3-HPA concentrations of 167±10 mM and 153±6 mM with and without N_2, respectively, were obtained after 45 min in glycerol solution. For lower initial biomass concentrations of $5.7\pm1.4\times10^{9}$ cfu/ml and $1.9\pm0.4\times10^{9}$ cfu/ml, maximal 3-HPA concentrations were obtained later (after 1 h and 2 h, respectively) and reached 149±7 mM and 82±8 mM with N_2 and 135±15 mM and 68±10 mM without N_2, respectively. As observed for other conditions, almost no 3-HPA was produced during the first 30 min of incubation for all conditions tested. Cell viability during incubation in glycerol dropped faster for increasing initial biomass concentrations, with no significant effect ($P>0.05$) of N_2 (Fig. 2b). No viable cells were detected by plate counts after 1.5 h of incubation for the highest initial cell concentration of $1.7\pm0.3\times10^{10}$ cfu/ml, whereas cell survivals for initial biomass concentrations of $5.7\pm1.4\times10^{9}$ cfu/ml and $1.9\pm0.4\times10^{9}$ cfu/ml averaged 3.8×10^{5} cfu/ml and 4.9×10^{7} cfu/ml after 2 h of incubation, respectively.

Effects of N_2 addition during glycerol bioconversion on 3-HPA production

The addition of N_2 during glycerol bioconversion to 3-HPA was also studied for three initial biomass concentrations ranging from $2.0\pm0.3\times10^{9}$ cfu/ml to $1.6\pm0.1\times10^{10}$ cfu/ml. For a given initial biomass concentration, 3-HPA productions and cell survivals with or without N_2 were not significantly different ($P>0.05$), but were higher for higher initial biomass concentrations, as observed for the effects of N_2 addition during cell growth (data not shown).

Effects of temperature during glycerol bioconversion on 3-HPA production

The effects of the temperature of incubation (ranging from 5°C to 37°C) during glycerol bioconversion to 3-HPA were studied in 200 mM glycerol with an initial biomass concentration of $1.7\pm0.3\times10^{10}$ cfu/ml. Maximum 3-HPA production (167±10 mM) was obtained at 30°C after

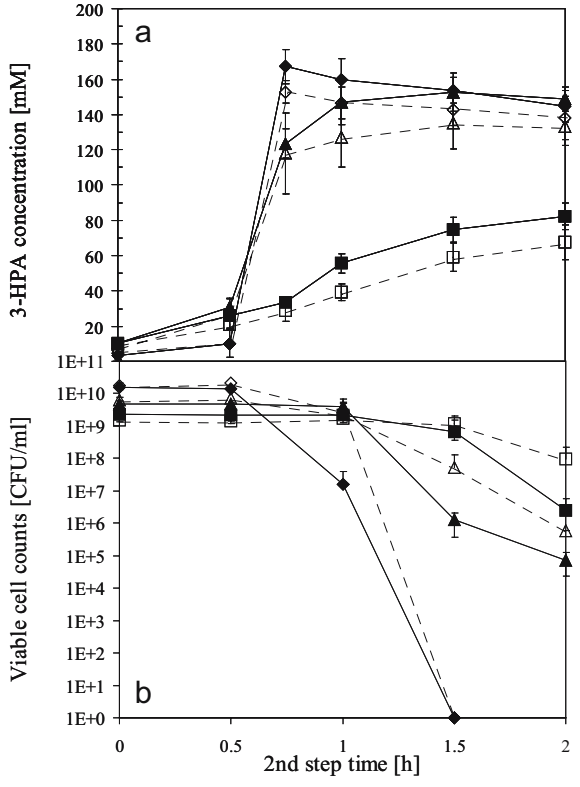

Fig. 2 Effects of anaerobic and micro-aerophilic conditions during the first biomass production step on: **a** 3-HPA production and **b** *Lb. reuteri* viability at 30°C and 200 mM glycerol for three different biomass concentrations (*filled symbols* with N_2, *open symbols* without N_2). *Squares* $1.9\pm0.4\times10^{9}$ cfu/ml, *triangles* $5.7\pm1.4\times10^{9}$ cfu/ml, *diamonds* $1.7\pm0.3\times10^{10}$ cfu/ml. *Error bars* on figures are standard deviations of duplicate experiments

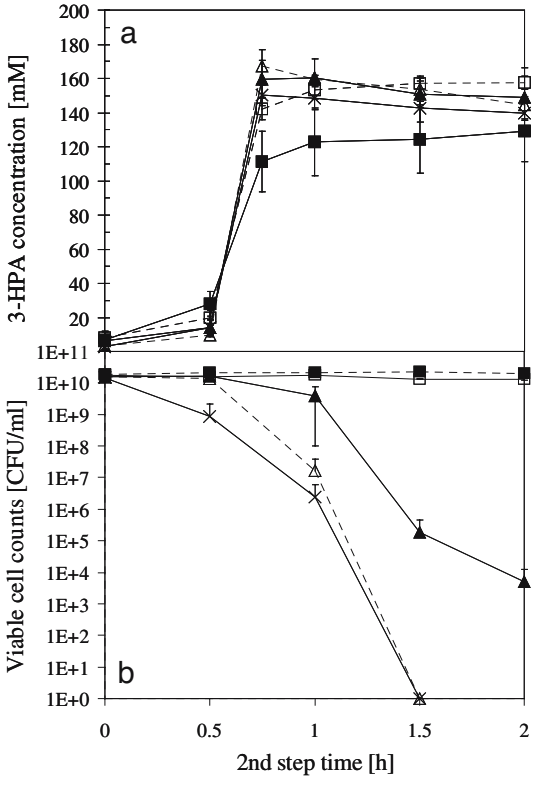

Fig. 3 Effects of temperature on: **a** 3-HPA production and **b** *Lb. reuteri* viability during the glycerol bioconversion step for an initial biomass concentration of $1.7\pm0.3\times10^{10}$ cfu/ml in 200 mM glycerol. *Filled squares* 5°C, *open squares* 15°C, *filled triangles* 23°C, *open triangles* 30°C, *crosses* 37°C

45 min, but a low temperature of 5°C still allowed a high production of 129±18 mM of 3-HPA after 2 h (Fig. 3a). No significant difference (P<0.05) in 3-HPA production was observed for temperatures between 15°C and 37°C. The effects of incubation temperature on *Lb. reuteri* viability were considerable, with low temperatures of 5°C and 15°C maintaining high cell viability close to 100% throughout the 2-h incubation period, whereas no cell survived at 30°C and 37°C after 1.5 h and a very low viable-cell concentration of 5.0×10^3 cfu/ml was measured at 23°C after 2 h (Fig. 3b).

Effects of glycerol concentration during glycerol bioconversion on 3-HPA production

The effects of glycerol concentration ranging from 200 mM to 400 mM on bioconversion to 3-HPA were studied for an initial biomass concentration of $1.6\pm0.3\times10^{10}$ cfu/ml and an incubation temperature of 30°C. High maximal 3-HPA production was reached after 45 min of incubation for all glycerol concentrations tested, corresponding to 167±10, 204±1, 224±19, and 235±3 mM in solutions containing 200, 250, 300, and 400 mM glycerol, respectively (Fig. 4a). After reaching a maximum of production after 45 min of incubation, 3-HPA concentrations remained stable throughout the 2-h experiments, except for 200 mM glycerol for which a slight and non-significant (P<0.05) decrease in 3-HPA concentration from 167±10 mM to 145±9 mM was observed. However, cell viability dropped drastically after 0.5–1.0 h of incubation in glycerol concentrations from 200 mM to 300 mM, with no cells detected on agar plates after 1.5 h (Fig. 4b). Surprisingly, cell viability in 400 mM glycerol dropped 1 h later compared with lower glycerol conditions, with no cells detected after 2 h.

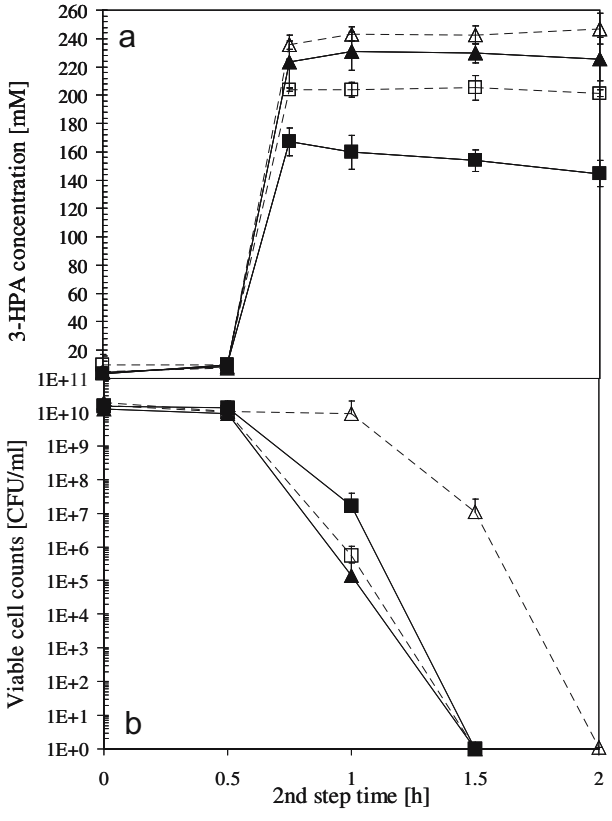

Fig. 4 Effects of glycerol concentration on: **a** 3-HPA production and **b** *Lb. reuteri* viability during the glycerol bioconversion step at 30°C for an initial biomass concentration of $1.6\pm0.3\times10^{10}$ cfu/ml. *Filled squares* 200 mM, *open squares* 250 mM, *filled triangles* 300 mM, *open triangles* 400 mM

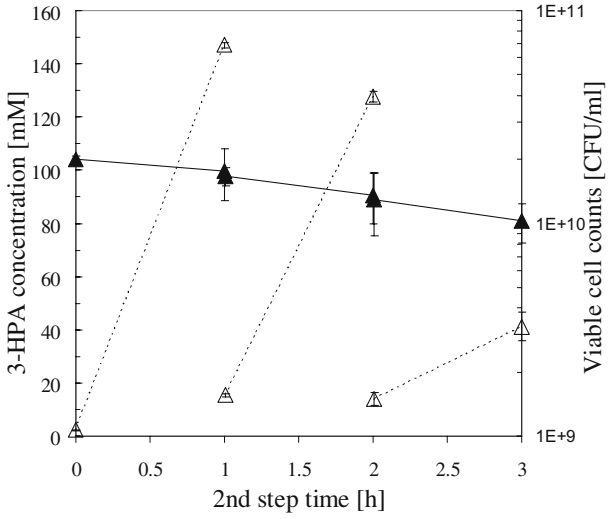

Fig. 5 3-HPA production (*open triangles*) and *Lb. reuteri* viability (*filled triangles*) during three successive cell incubations at 15°C in 200 mM glycerol. Biomass concentration at the beginning of the first incubation was $2.0\pm0.1\times10^{10}$ cfu/ml. After an initial incubation period (1 h) in glycerol, cells were harvested by centrifugation and resuspended in glycerol solution for 3-HPA production. This procedure was repeated twice

Successive incubations in glycerol

Three successive 1-h incubations in 200 mM glycerol were carried out at 15°C for an initial biomass concentration in the first incubation of $2.0\pm0.1\times10^{10}$ cfu/ml. 3-HPA production decreased after each incubation cycle, with final 3-HPA concentrations of 147±1, 128±2, and 41±5 mM corresponding to productions of 145±1, 112±1, and 28±2 mM determined at the end of the first, second, and third cycle, respectively (Fig. 5). Viable cell counts remained very high during the three incubations: $1.8\pm0.5\times10^{10}$, $1.4\pm0.4\times10^{10}$, and $1.0\pm0.2\times10^{10}$ cfu/ml at the end of the first, second, and third cycle, respectively.

Discussion

The aim of the present work was to optimize the production of 3-HPA by *Lb. reuteri* using a two-step process consisting of separate phases of biomass production and glycerol biotransformation to 3-HPA by resting cells. This strategy

was chosen since accumulation of 3-HPA, converted from glycerol by *Lb. reuteri*, can only occur in solutions containing no or very low levels of sugar, where 3-HPA reduction to 1,3-PDO (enabling the cells to recover the NAD+ used during glycolysis) does not occur (Vollenweider and Lacroix 2004). To get a better understanding of the dynamics of 3-HPA production by *Lb. reuteri*, 3-HPA concentration and cell viability were measured over time during each incubation of resting cells in glycerol solution. Indeed, it is well known that 3-HPA at a certain threshold concentration is toxic for the producing strain itself, which is a limiting factor for high 3-HPA production (Vollenweider and Lacroix 2004).

3-HPA accumulation occurred faster and increased with initial biomass concentration up to approximately 1.6×10^{10} cfu/ml, whereas higher cell concentrations appeared to inhibit 3-HPA accumulation (Fig. 1). This is surprising, since the sharpest drop in cell viability occurred for the highest biomass concentrations. These data could indicate the presence of high amounts of toxic 3-HPA that could not be detected, due to interactions with cellular material. Indeed, it has been shown that 3-HPA can react with free amino groups (Lüthi-Peng et al. 2002b; Sung et al. 2003). Therefore, higher initial cell concentrations can bind more 3-HPA, resulting in lower concentrations of free product. This might also explain the fact that after reaching a maximum 3-HPA accumulation after approximately 45 min of incubation for high initial biomass concentrations, 3-HPA concentration decreased slowly during prolonged incubation (Fig. 1).

3-HPA production immediately stopped after 45 min of incubation for initial biomass concentrations of 1.6×10^{10} cfu/ml or higher, which can be directly related to cell death caused by high 3-HPA accumulation. Cell death might be induced by the reaction of 3-HPA with redox-active molecules such as glutathione, thioredoxin, and glutaredoxin. It was thus shown in our laboratory that glutathione content almost disappeared in *Escherichia coli* cells killed by 3-HPA. However, the mode of action of 3-HPA is not yet known (Vollenweider and Lacroix 2004). In addition, all glycerol may have been metabolized, since a previous experiment carried out in 200 mM glycerol at 37°C with a initial biomass concentration of approximately 7.5×10^{9} cfu/ml reported a low residual glycerol concentration of only 5 mM after 2 h of incubation, with 3-HPA production of 170 mM (Lüthi-Peng et al. 2002b).

It was reported that anaerobic conditions are required for glycerol bioconversion into 3-HPA (Talarico et al. 1988; Talarico and Dobrogosz 1989; Font de Valdez et al. 1997). This led to the assumption that anaerobic conditions during cell growth and glycerol bioconversion might have a positive effect on 3-HPA production. Therefore, the effects of purging or not purging with N_2 the bioreactor headspace during biomass production and the incubation flask during glycerol bioconversion were studied to determine the impact of anaerobic/micro-aerophilic conditions on 3-HPA production during both steps. Sparging N_2 during cell growth did not have a major effect on 3-HPA production. However, maximal 3-HPA productions using cells grown anaerobically were 10–20% higher ($P<0.05$; for initial biomass concentrations ranging from $1.9\pm0.4\times10^{9}$ cfu/ml to $1.7\pm0.2\times10^{10}$ cfu/ml) than those obtained using cells propagated without N_2. The induction of glycerol dehydratase may thus be slightly inhibited by oxygen, which could result in a smaller amount of enzymes available for the glycerol bioconversion (Honda et al. 1980; Kajiura et al. 2001). Moreover, conditions in the bioreactor during cell growth carried out without adding N_2 are close to anaerobic conditions, due to the slight overpressure in the bioreactor resulting from the production of CO_2 by growing cells, which could also explain the small difference in 3-HPA production obtained when sparging or not the bioreactor with N_2. The effects of N_2 during glycerol bioconversion to 3-HPA were not significant ($P>0.05$) for all three initial biomass concentrations tested, indicating that glycerol dehydratase activity is probably not inhibited by oxygen.

As reported by Vollenweider and Lacroix (2004), 3-HPA can be converted into acrolein, a highly toxic α, β-unsaturated aldehyde, due to thermal dehydration; and the stability of 3-HPA in combination with sugars or free amino acids greatly depends on temperature, since the aldehyde function of 3-HPA reacts highly with sulfhydryl, hydroxyl, carboxyl, and amino acid functional groups (Chen et al. 2002; Lüthi-Peng et al. 2002b). This led to the assumption that 3-HPA may be less reactive at low temperatures, which might therefore improve 3-HPA yield or cell viability. The effects of incubation temperature between 5°C and 37°C on 3-HPA production and cell viability were thus tested during glycerol bioconversion for initial biomass and glycerol concentrations of $1.7\pm0.4\times10^{10}$ cfu/ml and 200 mM, respectively. No loss in viability occurred after 2-h incubations at 5°C and 15°C, whereas no viable cells were detected at 30°C and 37°C after 1.5 h, with high 3-HPA productions in all cases. This effect could be due to the decreased reactivity of 3-HPA at low temperatures, but could also be due to high cell sensitivity to 3-HPA at high temperatures close to the optimal growth temperature. The high productions obtained from 5°C to 37°C demonstrate the broad range of glycerol dehydratase activity responsible for glycerol bioconversion to 3-HPA. However, 3-HPA production stopped after 45 min at low temperatures, even though the cell viability remained unchanged. This could be due to the complete utilization of glycerol at this point.

The effects of glycerol concentrations from 200 mM to 400 mM were tested at 30°C and for an initial biomass concentration of $1.6\pm0.3\times10^{10}$ cfu/ml. A very high 3-HPA concentration of 247±11 mM could be obtained when 400 mM glycerol was used, which is the highest reported production of 3-HPA using *Lb. reuteri*. 3-HPA productions increased with glycerol concentration but always stopped after 45 min, in parallel with a sharp drop in cell viability at 30–60 min [except for 400 mM glycerol which apparently protected the cells during a longer period (60–90 min) against the toxic effects of 3-HPA]. The calculated conversion yields of glycerol to 3-HPA, assuming that all glycerol was used, were 84, 82, 77, and 62% for

glycerol concentrations of 200, 250, 300, and 400 mM, respectively. The fact that 3-HPA production at 300 mM and 400 mM glycerol was almost identical even though viable cells and probably glycerol were still present at 400 mM may indicate that glycerol dehydratase activity was inhibited either by high concentrations of glycerol, which has already been reported (Talarico and Dobrogosz 1990; Kajiura et al. 2001; Knietsch et al. 2003), or by high concentrations of 3-HPA, which could indicate in this case that the highest possible 3-HPA concentration was reached in the conditions used for the bioconversion step. However, the fact that cell viability, which is crucial for glycerol biotransformation to 3-HPA, decreased during incubation in 400 mM glycerol, could also explain the limited yield of 3-HPA.

In an attempt to reuse biomass, successive incubations in 200 mM glycerol for 1 h at 15°C were carried out with an initial biomass concentration of $2.0\pm0.1\times10^{10}$ cfu/ml, since high 3-HPA production and cell viability were obtained with these conditions (Fig. 3). During the first two cycles, 3-HPA productions were high (147±1 mM and 128±2 mM, respectively) and correlated well with viable cell counts, while cell viability decreased only slightly. Cell loss was mainly attributed to centrifugation and 3-HPA toxicity. Approximately 50% of the initial viable biomass concentration was tested at the end of the third incubation, whereas 3-HPA production dropped by approximately 80% compared with the first incubation cycle. It is thus expected that cells exhibited slightly altered glycerol metabolism during the third incubation, probably due to prolonged contact with high concentrations of 3-HPA and the starving conditions in the glycerol solution and centrifugation steps. It might then be recommended to add minute amounts of glucose to maintain cell activity without interfering with 3-HPA production. Another solution would be to use immobilized cells instead of free cells for glycerol bioconversion to 3-HPA, since this technology is very efficient for producing metabolites from lactic acid bacteria (Lacroix et al. 2005). Immobilized cells could be easily recovered for successive incubations in glycerol without the need for centrifugation steps; and phases of immobilized-cell activity regeneration could eventually be considered by incubating the biocatalysts in growth medium between 3-HPA production cycles in glycerol. Moreover, cells immobilized in gel beads were recently reported to exhibit an increased stress tolerance compared with free cells (Doleyres et al. 2004). Therefore, the use of immobilized cells of *Lb. reuteri* with increased tolerance to 3-HPA could represent a major advantage over free cells for producing 3-HPA.

The present study confirmed the high potential of *Lb. reuteri* ATCC 53608 for the biotechnological production of 3-HPA using a two-step process involving separate steps for biomass production and glycerol bioconversion to 3-HPA. 3-HPA production was largely dependent on biomass and glycerol concentrations and was maximal at room temperature (23–30°C), which is of great interest for industrial applications. However, 3-HPA at high concentrations appeared to be lethal for the producing strain at temperatures above 15°C. This problem could be avoided by using lower temperatures of incubation while maintaining high 3-HPA production. The reuse of cells for repeated production cycles could be limited by the loss of cell viability and alterations in glycerol metabolism. Work is currently underway to study the potential of immobilized cells to solve these limitations.

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