Biotechnol Lett (2014) 36:147–152
DOI 10.1007/s10529-013-1343-y

ORIGINAL RESEARCH PAPER

Elevated 3-hydroxypropionaldehyde production from glycerol using a *Citrobacter freundii* mutant

Thomas P. West · Jessica L. Peterson

Received: 22 June 2013 / Accepted: 5 September 2013 / Published online: 24 September 2013

Abstract A mutant strain of *Citrobacter freundii* capable of elevated 3-hydroxypropionaldehyde production from glycerol was isolated using chemical mutagenesis and a screening protocol. The protocol involved screening mutagenized bacterial cells on solid minimal medium containing 5 % (v/v) glycerol. Colonies were picked onto duplicate solid minimal medium plates and one plate was stained with 1 % (w/v) phloroglucinol. Those colonies staining red were further screened and a mutant, HPAO-1, was identified. The mutant strain produced a several-fold higher 3-hydroxypropionaldehyde concentration than did the parent strain when grown on 5 % (v/v) glycerol. The ratio of culture volume to flask volume influenced 3-hydroxypropionaldehyde production by the mutant cells compared to the parent cells. Aldehyde production was highest when the mutant strain was grown on 5 % (v/v) glycerol at a ratio of culture volume to flask volume of 1:3 or 1:12.5.

Keywords Biomass · *Citrobacter* · Glycerol · 3-Hydroxypropionaldehyde · Mutant · Phloroglucinol

T. P. West (✉) · J. L. Peterson
Department of Biology and Microbiology, South Dakota State University, Box 2104A, Brookings, SD 57007, USA
e-mail: thomas.west@sdstate.edu

Introduction

The primary industrial application for 3-hydroxypropionaldehyde is that it serves as a precursor for acrolein or acrylic acid utilized for plastic production (Vollenweider and Lacroix 2004). It can be used for DL-methionine production or as a food preservative (Vollenweider and Lacroix 2004). This aldehyde can also be converted to 1,3-propanediol which can be used as a polyester in textile fibers or thermoplastics (Vollenweider and Lacroix 2004; Liu et al. 2010). A number of enteric bacteria, including *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter agglomerans*, *Enterobacter aerogenes* and *Citrobacter freundii*, have been shown to contain the enzyme glycerol dehydratase which produces 3-hydroxypropionaldehyde from glycerol (Slininger et al. 1983; Slininger and Bothast 1985; Homann et al. 1990; Vancauwenberge et al. 1990; Boenigk et al. 1993; Barbirato et al. 1995, 1996a, b, 1998). Under optimum conditions in the presence of semicarbazide hydrochloride, *K. pneumoniae* NRRL B-4011 aerobically accumulated a high concentration of 3-hydroxypropionaldehyde from 7 % (v/v) glycerol (Vancauwenberge et al. 1990). Cells of *E. agglomerans* CNCM 120 grown anaerobically on 6.7 % glycerol produced 3-hydroxypropionaldehyde but at a much lower concentration than observed for *K. pneumoniae* NRRL B-4011 (Vancauwenberge et al. 1990; Barbirato et al. 1996a, b).

Citrobacter freundii can produce 3-hydroxypropionaldehyde when suspended in a neutral phosphate

buffer containing semicarbazide hydrochloride (an inhibitor of 1,3-propanediol oxidoreductase) and glycerol (Slininger et al. 1983; Ulmer and Zeng 2007). In continuous cultures of *C. freundii*, cell productivity was highest during glycerol limitation (Boenigk et al. 1993). In *C. freundii*, glycerol fermentation induced the dehydratase responsible for 3-hydroxypropionaldehyde production (Forage and Foster 1979). In this study, a mutant strain from *C. freundii* was isolated using a procedure that detected aldehyde production. It produced high amounts of 3-hydroxypropionaldehyde from glycerol compared to its parent strain ATCC 8090. The parent and mutant strains were investigated for their ability to produce 3-hydroxypropionaldehyde under selected culture conditions.

Materials and methods

Microorganism and medium

Citrobacter freundii ATCC 8090 was grown on culture medium (pH 7.1) consisting of KH_2PO_4/K_2HPO_4 (0.1 %, w/v), tryptone (0.5 %, w/v), yeast extract (0.5 %, w/v) and glycerol (2.5–10 %, v/v). During the semicarbazide hydrochloride optimization experiments, the phosphate buffer mixture (pH 7.4) contained potassium phosphate (1.74 %, w/v), glycerol (5 %, v/v) and semicarbazide hydrochloride (0.25–3 %, w/v). For the mutant isolation procedure, the phosphate buffer mixture (pH 7.4) contained KH_2PO_4/K_2HPO_4 (1.74 %, w/v), glycerol (5 %, v/v) and semicarbazide hydrochloride (2.68 %, w/v). Aeration studies used KH_2PO_4/K_2HPO_4 (1.74 %, w/v), glycerol (2.5–10 %, v/v) and semicarbazide hydrochloride (0.6 %, w/v).

Culture conditions used for 3-hydroxypropionaldehyde production

Shake-flask (250 ml Erlenmeyer flasks) cultures of the strains were grown in the glycerol-containing phosphate medium (50 ml) described above with aeration (150 rpm) for 24 h at 28 °C after being inoculated with each strain. The stationary phase cells of each strain were collected by centrifugation at $\sim 7{,}000 \times g$ for 20 min at 4 °C. The cells were resuspended into the appropriate phosphate buffer (pH 7.4) containing glycerol and semicarbazide hydrochloride as described above. The mixtures were shaken (150 rpm) in 15, 30 or 125 ml sterile Erlenmeyer flasks for 24 h at 28 °C and the cell suspensions were centrifuged at $\sim 7{,}000 \times g$ for 20 min at 4 °C. Following centrifugation, the 3-hydroxypropionaldehyde concentrations were measured in the supernatants while the cell pellets were used in the biomass determinations. The cellular biomass level used in the buffer containing glycerol and semicarbazide hydrochloride during the experiments was 12 g (dry weight) l^{-1}.

Mutant isolation procedure

Initially, ATCC 8090 cells were subjected to mutagenesis using 1.5 % (w/v) ethylmethane sulfonate for 75 min at 30 °C. The mutagenized cells were spread onto solid minimal medium A plates containing glycerol (5 %, v/v). The composition (%, w/v) of minimal medium A is 1.05 % KH_2PO_4, 0.45 % K_2H8O_4, 0.5 % $(NH_4)_2SO_4$, 0.25 % sodium citrate dihydrate and 2 % (w/v) agar. Colonies were picked onto duplicate solid minimal medium A plates and one plate was stained with 1 % (w/v) phloroglucinol (15 min at 50 °C). Colonies stained red by phloroglucinol were picked for further screening. Putative mutants and ATCC 8090 were grown on a culture medium (pH 7.1) that contained glycerol (5 %, v/v) and phosphate buffer (pH 7.4) as described above. The cultures were processed as indicated previously and the supernatants collected. After comparing the 3-hydroxypropionaldehyde levels produced by the possible mutants with the parent strain ATCC 8090, a mutant was identified as being capable of elevated production and was designated HPAO-1.

Effect of aeration on 3-hydroxypropionaldehyde production by strains

The mutant HPAO-1 and ATCC 8090 were grown on the culture medium (pH 7.1) described above containing glycerol (5 %, v/v). The cells were resuspended into a phosphate buffer (pH 7.4) mixture (10 ml) containing glycerol (5 %, v/v) and semicarbazide hydrochloride (0.6 %, w/v) as described above and the mixtures were shaken in 15, 30 or 125 ml sterile Erlenmeyer flasks for 24 h at 28 °C. When the effect of aeration upon mutant HPAO-1 production of

3-hydroxypropionaldehyde was explored relative to glycerol concentration, shake flask (50 ml) cultures of the strains were grown in the glycerol (2.5–10 %, v/v)-containing phosphate medium (50 ml) for 24 h at 28 °C. The cells were resuspended into a phosphate buffer (pH 7.4) mixture (10 ml) containing glycerol (2.5–10 %, v/v) and semicarbazide hydrochloride (0.6 %, w/v). The shake flask mixtures were shaken (150 rpm) in 30 ml or 125 ml sterile Erlenmeyer flasks for 24 h at 28 °C and processed to determine the 3-hydroxypropionaldehyde and biomass levels.

3-Hydroxypropionaldehyde and biomass determinations

The assay of 3-hydroxypropionaldehyde in the supernatants was done using the previously described procedure involving 3-methyl-2-benzothiazolinone that reacts to form a product that can be detected spectrophotometrically (Toraya et al. 1977; Barbirato et al. 1996b). The assay mixture (2.5 ml) contained 0.04 M potassium citrate buffer (pH 3.6) and 0.02 % (w/v) 3-methyl-2-benzothiazolinone hydrochloride and sample. After 15 min at 37 °C, the assay mixture was diluted with water (1 ml) and the absorbance of the mixture was measured at 305 nm (Toraya et al. 1977; Barbirato et al. 1996b). Biomass levels were determined gravimetrically where the bacterial cells were collected on preweighed filters, dried to constant weight at 80 °C and reweighed. Yield was based on the amount of 3-hydroxypropionaldehyde produced per gram glycerol present in the culture medium.

Results

Initially, it was necessary to determine under what incubation conditions using ATCC 8090 cells grown on 5 % (v/v) glycerol would the maximum level of 3-hydroxypropionaldehyde be observed. To observe the maximum 3-hydroxypropionaldehyde concentration, it is necessary to block its degradation using semicarbazide hydrochloride. Semicarbazide hydrochloride inhibits the bacterial oxidoreductase activity that converts 3-hydroxypropionaldehyde to 1,3-propanediol (Slininger et al. 1983; Slininger and Bothast 1985; Vancauwenberge et al. 1990). As seen in Fig. 1, a semicarbazide hydrochloride concentration of 0.6 % (w/v) results in a maximum concentration of

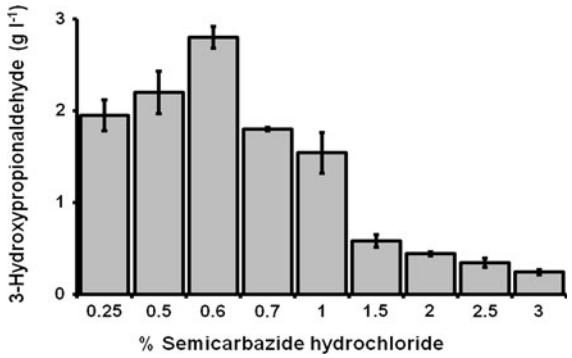

Fig. 1 3-Hydroxypropionaldehyde concentration (g l^{-1}) produced by ATCC 8090 cells grown on 5 % (v/v) glycerol and aerated in phosphate buffer containing 5 % (v/v) glycerol, selected concentrations of semicarbazide hydrochloride (%, w/v) and a biomass concentration of 12 g (dry weight) l^{-1}. The data represent the mean of three separate trials ± standard deviation (*error bars*)

3-hydroxypropionaldehyde being produced by the 5 % (v/v) glycerol-grown ATCC 8090 cells. Bacterial cell loss was not observed as the semicarbazide hydrochloride concentration was increased in the culture medium. A statistically significant difference ($P > 0.01$) in 3-hydroxypropionaldehyde production was noted at 0.6 % (w/v) semicarbazide hydrochloride compared to 0.5 or 0.7 % (w/v) semicarbazide hydrochloride (Fig. 1). This concentration was used in the subsequent characterization of the mutant properties.

The parent strain was subjected to chemical mutagenesis using ethylmethane sulfonate and the mutant cells were screened for their ability to produce aldehyde. The screening process involved staining putative mutant colonies with phloroglucinol and observing whether the colonies became stained with a reddish coloration following incubation with the dye (Gayathri and Balasubramanian 2000). Phloroglucinol reacts non-specifically with aldehydes producing a visible chromophore (Gayathri and Balasubramanian 2000). The colonies were screened for 3-hydroxypropionaldehyde production and the most effective aldehyde producer was designated HPAO-1. Next, the generation times of the parent strain and mutant strain were compared on the culture medium containing 5 % (v/v) glycerol. The generation time of ATCC 8090 (83 min) was more rapid than the mutant strain HPAO-1 (108 min). The mutant strain produced more than a 13-fold higher 3-hydroxypropionaldehyde level than ATCC 8090 when the ratio of the culture volume

(containing 5 % (v/v) glycerol, 0.6 % (w/v) semicarbazide hydrochloride and cells) to flask volume was 1:12.5. The difference in aldehyde production was found to be statistically significant ($P > 0.01$). The effect of decreasing the ratio of culture volume to flask volume on 3-hydroxypropionaldehyde production by the parent and mutant strains was investigated. Aldehyde production by the parent strain increased as the ratio of culture volume to flask volume decreased while mutant strain production decreased as the ratio decreased (Fig. 2). At a ratio of culture volume to flask volume of 1:3, the highest level of production by HPAO-1 and ATCC 8090 was noted (Fig. 2). Production of the aldehyde from glycerol by the mutant strain was 1.8-fold higher than its parent strain with the difference in production being statistically significant ($P > 0.01$). When the ratio of culture volume to flask volume was reduced to 1:1.5, the mutant strain produced slightly less 3-hydroxypropionaldehyde than its parent strain with no statistically significant difference in production being found (Fig. 2). Aeration was clearly a factor in 3-hydroxypropionaldehyde by both strains. The highest yield observed for ATCC 8090 from 5 % (v/v) glycerol was 52 % while it was 92 % for mutant strain HPAO-1 based upon the initial glycerol concentration in the culture medium.

Subsequently, we determined the optimal glycerol concentration for strain HPAO-1 to produce its highest level of 3-hydroxypropionaldehyde. The mutant strain was tested using a ratio of culture volume to flask volume of 1:3 and 1:12.5 because production by the strain was highest at these ratios. The highest 3-hydroxypropionaldehyde concentration produced by the mutant was with 5 % (v/v) glycerol using a ratio of culture volume to flask volume of 1:3 or 1:12.5 (Fig. 3). The presence of 2.5 or 10 % (v/v) glycerol in the buffer produced less 3-hydroxypropionaldehyde with the mutant strain when the ratio of culture volume to flask volume was 1:3 or 1:12.5 (Fig. 3). The difference in aldehyde production by the mutant strain on 5 % (v/v) glycerol was significantly higher ($P > 0.01$) than the concentrations produced by the strain on 2.5 or 10 % (v/v) glycerol when the ratio of culture volume to flask volume was 1:3 or 1:12.5. Statistically, aldehyde production by the mutant strain on 10 % (v/v) glycerol was significantly higher ($P > 0.01$) than on 2.5 % (v/v) glycerol independent of the ratio of culture to flask volume chosen. Based on the initial glycerol concentration in the culture medium, the highest yield of 3-hydroxypropionaldehyde produced by the mutant strain on 2.5 or 10 % (v/v) glycerol was 69 or 35 %, respectively. These yields were lower than the yield (92 %) produced by the mutant strain on 5 % (v/v) glycerol.

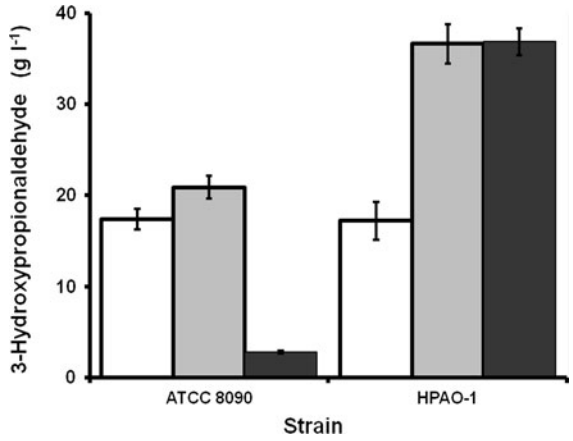

Fig. 2 3-Hydroxypropionaldehyde concentration (g l^{-1}) produced by ATCC 8090 and mutant HPAO-1 grown on 5 % (v/v) glycerol and aerated in phosphate buffer containing 5 % (v/v) glycerol, 0.6 % (w/v) semicarbazide hydrochloride and a biomass concentration of 12 g (dry weight) l^{-1} at a ratio of culture volume to flask volume of 1:1.5 (*white square*), 1:3 (*grey square*) or 1:12.5 (*black square*). The data represent the mean of three separate trials ± standard deviation (*error bars*)

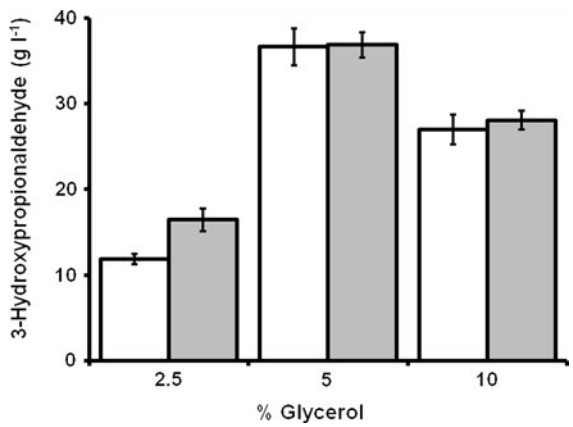

Fig. 3 3-Hydroxypropionaldehyde concentration (g l^{-1}) produced by mutant HPAO-1 grown on 2.5, 5 or 10 % (v/v) glycerol and aerated in phosphate buffer containing 2.5, 5 or 10 % (v/v) glycerol, 0.6 % (w/v) semicarbazide hydrochloride and a biomass concentration of 12 g (dry weight) l^{-1} at a ratio of culture volume to flask volume of 1:3 (*white square*), or 1:12.5 (*grey square*). The data represent the mean of three separate trials ± standard deviation (*error bars*)

Discussion

This study demonstrates that it is possible to isolate a *C. freundii* mutant which produces a higher 3-hydroxypropionaldehyde concentration than its parent strain by using a phloroglucinol-based screening protocol. The isolation of the mutant HPAO-1 is the first report of a mutant capable of producing a significantly higher 3-hydroxypropionaldehyde concentration compared to its parent strain using conventional mutagenesis and screening. Prior studies primarily have focused on the ability of species of *Klebsiella* and *Enterobacter* to produce the aldehyde from glycerol as well as exploring conditions that produce optimal aldehyde production. An early study using *K. oxytoca* cells found that the addition of 0.67 % (w/v) semicarbazide hydrochloride to the buffer allowed the cells to produce 3-hydroxypropionaldehyde from glycerol but increasing the semicarbazide hydrochloride concentration to 2.68 % (w/v) in the buffer significantly increased aldehyde production (Slininger et al. 1983). In contrast, the findings of this study indicated that a lower semicarbazide hydrochloride concentration was more effective in allowing ATCC 8090 cells to produce 3-hydroxypropionaldehyde from glycerol. When the semicarbazide hydrochloride concentration was increased from 0.6 to 3 % (w/v), 3-hydroxypropionaldehyde production by the cells steadily dropped. *K. oxytoca* ATCC 8724 produced up to 13.9 g 3-hydroxypropionaldehyde l^{-1} from 5 % (v/v) glycerol while *K. pneumoniae* NRRL B-4011 produced 46 g 3-hydroxypropionaldehyde l^{-1} from 7 % (v/v) glycerol (Slininger and Bothast 1985; Vancauwenberge et al. 1990). The decrease in 3-hydroxypropionaldehyde production by mutant HPAO-1 observed when the glycerol concentration was increased from 5 to 10 % (v/v) was similar to the drop in aldehyde production by *K. pneumoniae* when the glycerol concentration was increased from 5 to 9 % (Vancauwenberge et al. 1990). Using 6.7 % (v/v) glycerol, *K. pneumoniae* ATCC 15380 cells produced 54 g 3-hydroxypropionaldehyde l^{-1} after 11 h at 30 °C with an overall productivity of 3.5 g l^{-1} h^{-1} (Ulmer and Zeng 2007). The highest productivity observed in this study was 1.5 g l^{-1} h^{-1} indicating that strain HPAO-1 was less productive than *K. pneumoniae* cells in producing the aldehyde. *E. agglomerans* CNCM1210 was also less productive than the *Klebsiella* strains considering that CNCM 1210 cells produced only 2.2 g 3-hydroxypropionaldehyde l^{-1} when incubated with 6.68 % glycerol (Barbirato et al. 1996a).

Relative to *C. freundii*, anaerobic conditions supported 3-hydroxypropionaldehyde production (1.3 g l^{-1}) from 7 % glycerol (Barbirato et al. 1996a). Similarly, the findings here indicated that ATCC 8090 produced 3-hydroxypropionaldehyde from 5 % (v/v) glycerol and that increased aeration diminished its aldehyde production. In contrast, increased aeration elevated 3-hydroxypropionaldehyde production by the mutant. It was not clear why aeration had different effects on the parent and mutant strains. The mutant strain may exhibit increased 3-hydroxypropionaldehyde production due to elevated glycerol dehydratase activity, reduced 1,3-propanediol oxidoreductase activity or a reduced ability to regenerate NADH from NAD compared to the parent strain. Further work is needed to determine how glycerol metabolism is affected by the mutation in strain HPAO-1.

In conclusion, a protocol was devised that allowed the identification of a *C. freundii* mutant exhibiting elevated 3-hydroxypropionaldehyde production from glycerol. The mutant strain was as effective as wild-type strains of *K. pneumoniae* and *E. agglomerans* in producing 3-hydroxypropionaldehyde from glycerol.

Acknowledgments Financial support of this project by USDA NIFA Grant No. 2010-65504-20364 and the South Dakota Agricultural Experiment Station Grant SD00H198-07 was greatly appreciated.

References

Barbirato F, Camarasa-Claret C, Grivet JP, Bories A (1995) Glycerol fermentation by a new 1,3-propanediol-producing microorganism *Enterobacter agglomerans*. Appl Microbiol Biotechnol 43:786–793

Barbirato F, Grivet JP, Soucaille P, Bories A (1996a) 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. Appl Environ Microbiol 62:1448–1451

Barbirato F, Soucaille P, Bories A (1996b) Physiologic mechanism involved in accumulation of 3-hydroxy-propionaldehyde during fermentation of glycerol by *Enterobacter agglomerans*. Appl Environ Microbiol 62:4405–4409

Barbirato F, Soucaille P, Camarasa C, Bories A (1998) Uncoupled glycerol distribution as the origin of the accumulation of 3-hydroxypropionaldehyde during the fermentation of glycerol by *Enterobacter agglomerans* CNCM 1210. Biotechnol Bioeng 58:303–305

Boenigk R, Bowien S, Gottschalk G (1993) Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. Appl Microbiol Biotechnol 38:453–457

Forage RG, Foster MA (1979) Resolution of the coenzyme B-12-dependent dehydratases of *Klebsiella* sp. and *Citrobacter freundii*. Biochim Biophys Acta 569:249–258

Gayathri N, Balasubramanian N (2000) Spectrophotometric determination of formaldehyde. Anal Lett 33:3037–3050

Homann T, Tag C, Biebl H, Deckwer W-D, Schink B (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl Microbiol Biotechnol 33:121–126

Liu H, Xu Y, Zheng Z, Liu D (2010) 1,3-Propanediol and its copolymers: research, development and industrialization. Biotechnol J 5:1137–1148

Slininger PJ, Bothast RJ (1985) Optimizing aerobic conversion of glycerol to 3-hydroxypropionaldehyde. Appl Environ Microbiol 50:1444–1450

Slininger PJ, Bothast RJ, Smiley KL (1983) Production of 3-hydroxypropionaldehyde from glycerol. Appl Environ Microbiol 46:62–67

Toraya T, Ushio K, Fukui S, Hogenkamp HPC (1977) Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. J Biol Chem 252:963–970

Ulmer C, Zeng AP (2007) Microbial production of 3-hydroxypropionaldehyde from glycerol bioconversion. Chem Biochem Eng Q 21:321–326

Vancauwenberge JE, Slininger PJ, Bothast RJ (1990) Bacterial conversion to β-hydroxypropionaldehyde. Appl Environ Microbiol 56:329–332

Vollenweider S, Lacroix C (2004) 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. Appl Microbiol Biotechnol 64:16–27