Glycerol conversion to 1, 3-Propanediol is enhanced by the expression of a heterologous alcohol dehydrogenase gene in *Lactobacillus reuteri*.

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**Abstract**

In this work, *Lactobacillus reuteri* has been metabolically engineered for improving 1, 3-propanediol (1, 3-PD) production by the expression of an *Escherichia coli* alcohol dehydrogenase, *yqhD*, that is known to efficiently convert the precursor 3-hydroxypropionaldehyde (3-HPA) to 1, 3-PD. The engineered strain exhibited significantly altered formation rates for the product and other metabolites during the fermentation. An increase in the 1, 3-PD specific productivity of 34% and molar yield by 13% was achieved in the clone, relative to the native strain. A concomitant decrease in the levels of toxic intermediate, 3-HPA, was observed, with the specific productivity levels being 25% lesser than that of the native strain. Interestingly, the recombinant strain exhibited elevated rates of lactate and ethanol formation as well as reduced rate of acetate production, compared to the native strain. The preferential utilization of NADPH by YqhD with a possible decrease in the native 1, 3-PD oxidoreductase (NADH-dependent) activity, could have resulted in the diversion of surplus NADH towards increased lactate and ethanol productivities.

**Keywords:** 1, 3-propanediol oxidoreductase, YqhD, NADPH, 3-HPA, *L. reuteri*

**Introduction**

Biological processes are eco-friendly and sustainable alternatives to conventional chemical processes for production of several industrially important bulk chemicals like succinic acid, lactic acid, 1, 3-propanediol, 1, 4-butanediol, etc. (Biebl et al. 1998; Chotani et al. 2000; Song and Lee 2006). Such processes could be economically viable if they are based on renewable feedstocks. Glycerol, a surplus byproduct of the biodiesel industry holds promise as a major feedstock for synthesis of platform chemicals such as 1, 3-propanediol (Zhu et al. 2002). Currently, 1, 3-propanediol (1, 3-PD) has attracted worldwide interest due to its enormous applications in polymers, cosmetics, foods, adhesives, lubricants, laminates, solvents, antifreeze and medicines (Homann et al. 1990; Colin et al. 2000; Zhu et al. 2002; Cheng et al. 2007).

The biological route involves 1, 3-PD production by microorganisms like *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridia* and *Lactobacilli* (Biebl et al. 1999; Saxena et al. 2009). Amongst these, *Clostridium butyricum* and *Klebsiella pneumoniae*, are considered to be the best producers (Gonzalez-Pajuelo et al. 2006). 1, 3-PD concentrations in the range of around 40 - 100 g/l have been obtained with these producers (Celinska 2010). The product levels of the native producers have been improved using various bioprocess strategies. Metabolic engineering is currently being attempted to further enhance the product levels (Saxena et al. 2009).

The non-native producers, *Escherichia coli* and *Saccharomyces cerevisiae*, have also been engineered for 1, 3-PD production. In *S. cerevisiae*, due to ineffective transport of vitamin B12 needed for 1, 3-PD synthesis, only low levels of the product has been obtained. On
the other hand, *E. coli* has been metabolically engineered by DuPont and Genencor International, Inc., to produce 1,3-PD at a concentration of 135 g/l, (Maervoet et al. 2011) the highest reported so far in the industry. A major concern with the existing 1,3-PD producers is that a majority of them are opportunistic pathogens, that are less suitable for niche applications in food, cosmetic and biomedical industries. In this context *Lactobacillus reuteri*, a GRAS (generally regarded as safe) organism, offers immense potential as a host for 1,3-PD production.

*Lactobacillus reuteri* converts glycerol to 1,3-PD in a two-step anaerobic process (Figure 1). In the first step, a cobalamin-dependent glycerol dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA). In the second step, 3-HPA is reduced to 1,3-PD by a NADH-dependent oxidoreductase (Talarico et al. 1990). 1,3-PD productivity of around 10-30 g/l has been achieved so far in native *L. reuteri* (Baeza-Jimenez et al. 2011; Tobajas et al. 2009).

The major bottleneck limiting 1,3-PD production in *L. reuteri* is growth inhibition by secreted metabolites and toxic 3-HPA. These metabolites are produced to regenerate the cofactors such as NADH/NADPH. Therefore redirecting flux from these competing pathways towards product formation by balancing the redox potential would be a powerful metabolic engineering strategy. For instance, disruption of ethanol synthesis has been demonstrated to substantially improve flux through the 1,3-PD biosynthetic pathway in *K. pneumoniae* (Zhang et al. 2006). Further, redirection of flux from central carbon metabolism towards 1,3-PD synthesis should be complemented by adequate levels of enzymes and cofactors involved in the pathway.

In this work, we have expressed an *E. coli* alcohol dehydrogenase, *yqhD*, in *L. reuteri*, to increase 1,3-PD productivity by improved conversion of 3-HPA. Further, the impact of the heterologously expressed *yqhD* on cell growth, 1,3-PD production and byproduct formation has been analyzed.

**Figure 1** Pathways of glucose and glycerol metabolism in *L. reuteri*. Abbreviations: G6P glucose-6-phosphate, 6PG 6-phosphogluconate, R5P ribulose-5-phosphate, X5P xylulose-5-phosphate, AcP acetyl phosphate, AcCoA acetyl-CoA, F6P fructose-6-phosphate, FBP fructose-1,6-bisphosphate, DHAP dihydroxyacetone phosphate, GAP glyceraldehyde-3-phosphate, Pyr pyruvate, G3P glyceral-3-phosphate, 3-HPA 3-hydroxypropionaldehyde, GDHt glycerol dehydratase, 1,3-PDOR 1,3-propanediol oxidoreductase in *L. reuteri*, YqhD *E. coli* alcohol dehydrogenase.
Table 1 Bacterial strains and plasmid vectors used in this work

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| E. coli DH5α      | Cloning host for TA vector | Invitrogen, USA |
| E. coli EC1000    | Cloning host for pSIP411 | Dr Jan Kok, University of Groningen, Netherlands |
| RBC- TA vector    | TA cloning vector | RBC Bioscience Corp., Taiwan |
| pSIP411           | E. coli-lactobacillus shuttle expression vector | Servig et al. (2005) |
| L. reuteri ATCC55730 | Host | Biogaia, Sweden |
| L. reuteri HR2    | L. reuteri with yqhD | This study |
| E. coli K-12 MG1655 | Source of yqhD gene | Prof. Takashi Horiuchi, National Institute for Basic Biology, Japan. |
| pH1R              | TA vector with yqhD | This study |
| pH2R              | pSIP411 with yqhD | This study |

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used and modified in this study are listed in Table 1.

Media and growth conditions

*L. reuteri* ATCC 55730 and the *E. coli* strains were grown at 37°C in MRS (MRS contains 5 g yeast extract, 10 g proteose peptone, 10 g beef extract, 2 g dipotassium phosphate, 2 g ammonium citrate, 5 g sodium acetate, 100 mg magnesium sulphate, 50 mg manganese sulphate, 1 g polysorbate 80 and 20 g dextrose, per liter) broth and LB broth, respectively. The recombinants were cultured in media containing appropriate antibiotics, ampicillin (100 μg/ml) and erythromycin (200 μg/ml for *E. coli* and 5 μg/ml for *L. reuteri*). Growth was monitored by measuring the absorbance at 600 nm. Cell dry weight (CDW) was calculated from a predetermined relationship between *L. reuteri* CDW and optical density (1 OD₆₀₀ corresponded to 0.33 g/l CDW).

Chemicals and Reagents

The enzymes and reagents used in cloning experiments - *Neol*, *Xhol*, T4 DNA ligase, and Phusion™ Flash High-Fidelity PCR Master Mix - were bought from New England Biolabs (Manassas, USA). Plasmid miniprep spin kit and PCR purification kit were procured from Qiagen (Germany). Primers were procured from VBC-Biotech (Austria) and the inducer sakacin P induction peptide (SppIP) was synthesized from GenScript (USA). Culture media (LB and MRS), the antibiotics, erythromycin and ampicillin, and other chemicals were purchased from HiMedia Laboratories (Mumbai, India). Since 3-HPA standard could not be commercially procured, it was synthesized in our laboratory as described under “3-HPA production by resting cells of *L. reuteri* ATCC 55730”.

Construction of the recombinant plasmids

A schematic representation of the structure of recombinant plasmid, pH2R, carrying *yqhD*, is shown in Figure 2. The 1.163 kb *yqhD* gene fragment (GenBank accession number NC010498), was amplified from the chromosomal DNA of *E. coli* K-12 MG1655 using the primers *yqhDF* and *yqhDR* (Table 2). PCR conditions employed were - an initial denaturation at 98°C (10 s), followed by 25 cycles of the program: 98°C (3 s); 65°C (5 s); 72°C (20 s) and a final extension at 72°C (1 min).

The amplicon was cloned into TA vector to generate the recombinant plasmid pH1R. Further, the *yqhD* gene was sub-cloned from pH1R into *Neol*/Xhol site of pSIP411, resulting in recombinant plasmid, pH2R. The clones were screened by lysate PCR using the primer pair PorFXF and yqhDR (Table 2). The plasmid pH2R was electroporated into *L. reuteri* to yield, *L. reuteri* HR2. The electrocompetent cells were prepared as described by Berthier et al. (1996). Electroporation was performed with a BTX electroporator, using pulse settings of 1.5 kV, 800 Ω and 25 μF and a time constant of 11 - 13 ms was obtained. The cells were plated on MRS agar containing the required antibiotic and incubated for 24 - 36 h at 37°C until visible colonies were observed. The recombinant plasmid pH2R was isolated from *L. reuteri* HR2 using the plasmid miniprep kit, with the following modifications: The cells in resuspension buffer, were lysed with 30 mg/mL lysozyme (USB) and incubated at 37°C for 30 minutes. The rest of the procedure was as per the miniprep manual (Qiagen).

Batch fermentation

The inoculum for the batch reactor was grown in 150 mL MRS broth with erythromycin at 37°C until an OD₆₀₀ of 0.8 - 1.0 was reached. The seed was then inoculated into a 2 L fermentor (KLF 2000 - Bioengineering AG, Switzerland) filled with 1.2 L MRS medium containing erythromycin and glycerol (278 mM). A glucose to glycerol ratio of 1:2.5 has been used in this study for elevated 1, 3-PD synthesis (Tobajas et al. 2009). Fermentation was carried out at 37°C and 250 rpm, in an anaerobic condition. The pH was maintained at 5.5 by the addition of 1.5 M NaOH or 1.5 M H₃PO₄ (El-Ziney et al. 1998). The anaerobic condition was established by flushing with sterile nitrogen. At 0.8 OD₆₀₀, the culture was induced with 50 ng/mL of sakacin P induction peptide (SppIP). Samples were
removed periodically for determining OD_{600}. The culture pellet and supernatant were stored at -20°C, to be used later for protein and metabolite analyses respectively.

Substrate and Metabolite Analyses
Concentrations of glucose, glycerol, 1,3-PD, ethanol, lactate, 3-HPA and acetate in the culture broth were determined using an HPLC (Shimadzu LC-10AT VP) that was equipped with a refractive index detector (RID) and an aminex HPX-87H column (300 × 78 mm, Bio-Rad, USA). The mobile phase consisted of acetonitrile and water in a ratio of 35:65 in 5 mM H_2SO_4, at 0.4 mL/min. The temperature of column and RID was maintained at 30°C and 50°C respectively. Samples were filtered through 0.22 μm filters before analysis. 3-HPA standard was synthesized in the lab using resting cells of \textit{L. reuteri} ATCC 55730 as explained below. Quantitation of 3-HPA was done by HPLC, as described by Spinler et al. (2008).

3-HPA production by resting cells of \textit{L. reuteri} ATCC 55730
3-HPA was produced as described previously (Spinler et al. 2008; Luthi-Peng et al. 2002). Briefly, \textit{L. reuteri} was cultured in 100 mL MRS broth, incubated anaerobically at 37°C for 24 h. The anaerobic condition was maintained by sparging with nitrogen. The culture was centrifuged and the pellet washed with 50 mM sodium phosphate buffer (pH 7.4). The cells were resuspended in 250 mM glycerol to a concentration of ~1.5 × 10^{10} cells/mL and incubated anaerobically at 37°C for 2 h. After the 2 h incubation, the culture was pelleted and the 3-HPA-containing supernatant was collected and filter-sterilized using a 0.22 μm filter and the filtrate used for HPLC analysis.

SDS-PAGE analysis of yqhD expression in \textit{L. reuteri}
The SDS-PAGE was conducted on a 12% polyacrylamide gel (Laemmli 1970). The proteins on the gel were stained with 0.025% (w/v) Coomassie Brilliant Blue G-250. Protein concentration was determined by the Bradford method (Bradford 1976) with bovine serum albumin (BSA) as standard.

Results
Heterologous expression of alcohol dehydrogenase (yqhD) in \textit{Lactobacillus reuteri} ATCC 55730
The \textit{E. coli} alcohol dehydrogenase gene (yqhD) was cloned and expressed in \textit{L. reuteri}. The recombinant
plasmid, pH2 with yqhD gene was constructed as shown in Figure 2. The expression of the cloned yqhD gene in L. reuteri was confirmed using SDS-PAGE analysis of whole cell lysates (Figure 3). A prominent band of ~43 kDa appeared in the recombinant cells after induction, which correlates well with the expected size of YqhD.

**Batch fermentation analysis of recombinant L. reuteri harbouring yqhD**

To investigate the impact of yqhD expression on cell growth, substrate consumption, formation of 1, 3-PD, 3-HPA and other metabolites, batch fermentation of recombinant L. reuteri was carried out, with native strain as control. The cell concentration of both native and recombinant strains reached around 1.8 and 1.4 g/l of CDW respectively. The specific growth rate ($\mu_{\text{max}}$) of the recombinant strain was lower (0.38 h$^{-1}$) compared to the wild type (0.46 h$^{-1}$) (Figure 4).

It was observed that yqhD expression in L. reuteri altered the specific substrate uptake, product and byproduct formation rates significantly (Figure 5). The specific production rate of 1.38 g/g h for 1, 3-PD in the recombinant strain achieved during the log phase after induction, was notably higher (by 34%) than that of the native strain (1.03 g/g h) (Figure 5). This correlates with a 25% decrease in the levels of 3-HPA secreted in the recombinant cells after induction, which correlates well with the expected size of YqhD.

![Figure 3 SDS-PAGE analysis of L. reuteri whole cell lysates for yqhD expression. Lane 2 untransformed L. reuteri, lane 3 uninduced recombinant L. reuteri HR2, lane 4 recombinant 5 h after induction with SppIP, lanes 1 & 5, protein molecular weight marker.](image)

Table 3. Specific rates of formation of NADPH and acetate, lactate, ethanol and acetate in recombinant and native L. reuteri strains.

| Carbon Source | Recombinant | Native |
|---------------|-------------|--------|
| NADPH (mmol/g h) | 29.8 | 22.5 |
| Acetate (mmol/g h) | 3.1 | 2.0 |
| Lactate (mmol/g h) | 2.8 | 0.3 |
| Ethanol (mmol/g h) | 1.2 | 0.5 |

**Discussion**

L. reuteri produces 1, 3-PD along with 3-HPA only when glycerol is cofermented with glucose. Lower glucose levels have been shown to favour 3-HPA formation. Higher glucose concentrations generate more NADH, that is consumed for reducing 3-HPA to 1, 3-PD. Glycerol serves as an electron sink by recycling NADH produced during glycolysis (Luthi-Peng et al. 2002; Schutz and Radler 1984). In this work, 1, 3-PD synthesis is observed both in native and recombinant strains only when both the carbon sources are utilized (Figure 4, 6b). In the case of native strain, glycerol consumption upon exhaustion of glucose resulted in 3-HPA accumulation, since NADH supply could be limited by reduced glycolysis. Thus redox balance plays a crucial role in 1, 3-PD formation.

Enhancing the enzyme concentration and cofactor availability could lead to improved 1, 3-PD formation. As the phosphoketolase pathway prevalent in L. reuteri (Årsköld et al. 2008), provides increased NADPH, overexpression of yqhD, has the potential to further improve 1, 3-PD productivity. In this work, expression of yqhD has increased the molar yield of 1, 3-PD from glycerol by 13% in L. reuteri HR2. This is in contrast to the results reported by Zhuge et al. (2010) in recombinant K. pneumoniae strain, wherein yqhD overexpression did not increase the 1, 3-PD yield. However, upon overexpression of yqhD, they have observed a reduction in the activity of the native 1, 3-PD oxidoreductase (1, 3...
PDOR), with increased ethanol production. A similar diminishing activity of the native 1, 3 PDOR is perceived in \textit{L. reuteri} HR2, along with elevated rates of lactate and ethanol production.

The enhanced formation rates of lactate and ethanol observed in the recombinant \textit{L. reuteri} strain could be indirectly linked to the preferential utilization of NADPH by YqhD for 3-HPA conversion. The consumption of NADPH by YqhD and a possible reduction in the native NADH-dependent 1, 3-PDOR activity could have led to an increased cellular NADH/NAD$^+$ ratio. The surplus NADH thus generated has been diverted for the production of NADH-consuming metabolites like lactate and ethanol.

The elevated specific production rate of ethanol with concomitant decrease in specific acetate production rate implies that acetyl phosphate is channeled more towards ethanol production (Figure 5). This is most likely reflected as a shift in metabolism from acetate to ethanol production, resulting in reduced ATP synthesis. The

![Figure 4](image1.png)

**Figure 4** Time course of glucose (---), glycerol (---) consumption and biomass (-----) growth in native (triangles) and recombinant (open circles) \textit{L. reuteri} strains during batch cultivation.

![Figure 5](image2.png)

**Figure 5** Specific rates of substrate uptake and product formation in the logarithmic phase of batch fermentation using native (white bar) and recombinant \textit{Lactobacillus reuteri} (black bar) strains.

| Glycerol consumed (g/l) | 1, 3-propanediol produced (g/l) | Molar yield (mol/mol) |
|-------------------------|-------------------------------|----------------------|
| \textit{L. reuteri} ATCC 55730 | 30.02 | 11.0 | 0.45 |
| \textit{L. reuteri} HR2 | 21.6 | 9.1 | 0.51 |

Table 3 Comparison of 1, 3-PD molar yield of wild type and recombinant \textit{L. reuteri} in batch fermentation.
decreased ATP production coupled with the diversion of NADPH away from biosynthesis by YqhD, could have contributed to the decreased growth rate of the recombinant culture (Jarboe et al. 2010; Zhu et al. 2009). The decreased $\mu_{\text{max}}$ of the recombinant strain could also be attributed to the metabolic load imposed by the recombinant plasmid on the host (Bentley et al. 1990). Further, metabolic flux analysis needs to be carried out by measuring the enzyme activities and cofactors to verify this hypothesis. The present work has indicated that metabolic engineering can be effectively used to enhance 1, 3-PD productivity in L. reuteri. Further engineering of the strain to improve the redox balance and minimize the formation of byproducts like lactate and ethanol could pave the way for maximizing 1, 3-PD biosynthesis.

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Competing interests
The authors declare that they have no competing interests.

References

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Figure 6 Time course of metabolite formation by recombinant (open circles) and native strain (triangles) strains of L. reuteri in batch cultivation. a lactate (— —), acetate (— —) and ethanol (— —). b 1, 3-propanediol (— —) and 3-HPA (— —).
during glucose/glycerol cofermentation in batch and continuous cultures. Biotechnol Lett 20:913–916. doi:10.1023/A:1005434316757.

Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Soucaille P, Vasconcelos I (2006) Microbial conversion of glycerol to 1,3-propanediol: physiological comparison of a natural producer, Clostridium butyricum VPI 5286, and an engineered strain, Clostridium acetobutylicum DGIc0P0S. Appl Environ Microbiol 72(1):96–101. doi:10.1128/AEM.72.1.96-101.2006.

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

Jarboe LR (2010) YqhD: a broad-substrate range aldehyde reductase with various applications in production of biorenewable fuels and chemicals. Appl Microbiol Biotechnol 10:2912–2919.

Lüthi-Peng Q, Dileme FB, Puhan Z (2002) Effect of glucose on glycerol bioconversion by Lactobacillus reuteri. Appl Microbiol Biotechnol 59:289–296. doi:10.1007/s00253-002-1002-z.

Schutz H, Radler F (1984) Anaerobic reduction of glycerol to 1,3-propanediol by Lactobacillus brevis and Lactobacillus buchneri. Syst Appl Microbiol 5:169–178.

Song H, Lee SY (2006) Production of succinic acid by bacterial fermentation. Enzyme Microb Technol 39:352–361. doi:10.1016/j.enzmictec.2005.11.043.

Zhu MM, Lawman PD, Cameron DC (2002) Improving 1,3-propanediol production from glycerol in a metabolically engineered Escherichia coli by reducing accumulation of sn-glycerol-3-phosphate. Biotechnol Prog 18:694–699. doi:10.1021/bp020281+. Zhuge B, Zhang C, Fang H, Zhuge J, Permaul K (2010) Expression of 1,3-propanediol oxidoreductase and its isozyme in Klebsiella pneumoniae for bioconversion of glycerol into 1,3-propanediol. Appl Microbiol Biotechnol 87:2177–2184. doi:10.1007/s00253-010-2678-0.

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