0273-2289/92/3435—0149$02.20

Enhancement of 1,3-Propanediol Production by Cofermentation in *Escherichia coli* Expressing *Klebsiella pneumoniae dha* Regulon Genes

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ABSTRACT

1,3-Propanediol (1,3-PD) is an intermediate in chemical and polymer synthesis. We have previously expressed the genes of a biochemical pathway responsible for 1,3-PD production, the *dha* regulon of *Klebsiella pneumoniae*, in *Escherichia coli*. An analysis of the maximum theoretical yield of 1,3-PD from glycerol indicates that the yield can be improved by the cofermentation of sugars, provided that kinetic constraints are overcome. The yield of 1,3-PD from glycerol was improved from 0.46 mol/mol with glycerol alone to 0.63 mol/mol with glucose cofermentation and 0.55 mol/mol with xylose cofermentation. The engineered *E. coli* also provides a model system for the study of metabolic pathway engineering.

Index Entries: 1,3-Propanediol, *Escherichia coli*; cofermentation; *dha* regulon; metabolic pathway engineering.

INTRODUCTION

1,3-Propanediol (1,3-PD, also trimethylene glycol) is a chemical intermediate used in organic synthesis and as a chain extender in polyurethanes and polyesters. The current industrial production of 1,3-PD is derived from acrolein, a petroleum derivative. The motivation for our work is threefold:

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1. To improve the microbial fermentation of glycerol to 1,3-PD;
2. To extend the substrate range of the 1,3-PD fermentation to sugars and starch; and
3. To use the 1,3-PD pathway as a model system to develop tools and methodologies for metabolic pathway engineering.

1,3-PD was recognized as a byproduct of the Connstein and Lüdecke yeast glycerol fermentation in the early 1920s (*1*); the production from glycerol in pure culture was demonstrated in the 1930s and 1940s (*2,3*). The biochemical pathway for the production of 1,3-PD from glycerol has been studied primarily in *Klebsiella pneumoniae.* Glycerol is passively transported into the cell by the glycerol facilitator (*4*) and is then converted into 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B_{12}-dependent dehydratase (*5*). The 3-HPA is reduced to 1,3-PD by an NADH-dependent 1,3-propanediol oxidoreductase (*6*). 1,3-PD is then excreted into the medium (*7*).

The 1,3-PD pathway in *K. pneumoniae* is part of the *dha* regulon, which is induced by dihydroxyacetone (DHA) (*7*). The other two enzymes of the *dha* regulon (the DHA branch of the *dha* regulon) convert glycerol to DHA by an NAD^+-dependent glycerol dehydrogenase (*8*) and then to dihydroxyacetone phosphate (DHAP) by an ATP-dependent DHA kinase (*9*); the DHAP is further metabolized to provide carbon and energy for growth and maintenance. The physiological reason for 1,3-PD formation is most likely to regenerate NAD^+ needed by the DHA branch of the *dha* regulon (*10*).

We have constructed a cosmid, pTC1, with genes from the *K. pneumoniae dha* regulon and expressed the genes in *E. coli* (wild-type *E. coli* does not have a *dha* regulon) (*11*). The transformed *E. coli* AG1/pTC1 produces 1,3-PD from glycerol under anaerobic conditions. In this article, we describe the enhancement of the production of 1,3-PD from glycerol by cofermentation with glucose and with xylose.

MATERIALS AND METHODS

Bacteria, Cosmid, Media, and Growth Conditions

E. coli AG1 (*F*$^-$, *endA1, hsdR17*[k_n^-, m_k^+], *supE44, thi-1, recA1, gyrA96, relA1,* λ^-) (Stratagene, La Jolla, CA) was used as the host strain for the genomic library. The construction of cosmid pTC1 has been described previously (*11*). Anaerobic fermentations were carried out in 300-mL anaerobic flasks (*12*). Unless otherwise specified, growth experiments were done at 37°C with modified ST medium (Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NH_4Cl, 2 g/L; NaCl, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 2 m*M*; thiamine, 0.5 mg/L; coenzyme B_{12}, 0.5 mg/L; $FeSO_4 \cdot 7H_2O$, 0.278 mg/L; $ZnCl_2$, 0.136 mg/L; $CaCl_2 \cdot 2H_2O$, 1.47 mg/L, and cysteine-HCl·H_2O, 0.5 g/L) plus the appropriate carbon source(s) and antibiotic(s). All fermentations were supplied

with 1% yeast extract to ensure good growth and 100 μg/mL ampicillin to stabilize the cosmid pTC1. Anaerobic growth on agar plates was done in sealed jars under an H_2/CO_2 atmosphere (GasPak Anaerobic System, Becton Dickinson and Co., Cockeysville, MD).

HPLC Analysis

All fermentation samples were centrifuged and filtered through a 0.45-μm filter before analysis. 1,3-PD, ethanol, and organic acids were analyzed by HPLC (Bio-Rad Laboratories, Richmond, CA) with an organic acids column (Bio-Rad HPX87H) using the following conditions: sample size, 20 μL; mobile phase, 0.01*N* H_2SO_4; flow rate, 0.5 mL/min; column temperature, 40°C; detector, refractive index at room temperature. Sugars and glycerol were analyzed using a Waters Model 600 HPLC (Milford, MA) with a cation-exchange column in the calcium form (Waters Sugar-Pak II) under the following conditions: sample size, 10 μL; mobile phase, deionized water; flow rate, 0.5 mL/min; column temperature, 90°C; detector, refractive index at 35°C.

Determination of D and L-Lactate

The concentration of L-lactate in fermentation samples was measured with an enzymatic L-lactate analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). The analyzer was calibrated with a standard of 0.45 g/L L-lactate. D-Lactate concentration was estimated from the difference between the total lactate concentration measured by HPLC and the L-lactate value from the enzymatic analyzer.

RESULTS

The results of cofermentations of glycerol and sugar (glucose or xylose) by *E. coli* AG1/pTC1 at 30 h are summarized in Tables 1 and 2. Control fermentations of glycerol, sugars, and yeast extract alone are also shown. No 1,3-PD was detected without glycerol present. 1,3-PD yields, concentrations, and productivities were higher in the cofermentation cases than with fermentation of glycerol alone. The highest yield and concentration were achieved by the cofermentation of glycerol and glucose. Cell growth was significantly better when sugars were present than in the glycerol or yeast extract control. The relatively low cell density on glycerol is not particularly surprising since wild-type *E. coli* does not grow at all on glycerol without an exogenous electron acceptor, such as oxygen, fumarate, or nitrate.

Formate, lactate, and acetate were the dominant byproducts in all the fermentations, except in the yeast extract control (Run 6), where the succinate concentration was greater than the lactate concentration (Table 2).

Table 1
Summary of Final Values for Cofermentations and Control Fermentations

Run #	Carbon sources[a]	1,3-PD concentration, g/L	1,3-PD yield, mol/mol[b]	Maximum productivity, g/g/h[c]	Final cell mass, OD @ 660 nm	Carbon recovery, %[d]
1	Glycerol + glucose	1.20	0.63	1.42	0.95	105.6
2	Glycerol + xylose	1.08	0.55	1.41	1.00	94.4
3	Glycerol[e]	0.67	0.46	1.07	0.65	113.9
4	Xylose	N.D.[g]	-	-	0.98	101.8
5	Glucose	N.D.[g]	-	-	0.94	96.3
6	None[f]	N.D.[g]	-	-	0.55	-

[a] All fermentations contain 10 g/L yeast extract and were incubated at 37°C for 30 h.
[b] Mol 1,3-PD/mol glycerol consumed.
[c] g 1,3-propanediol/g cell mass/h (assuming 1.0 OD is equivalent to 0.5 g dry cell wt/L). The log-mean average cell density was used for calculation between two data points.
[d] Carbon recovery was corrected for yeast extract consumption by subtracting the cell mass and byproduct values of control Run 6 from the values of Runs 1–5 (the formula "mol" wt of *E. coli* was taken to be 24.5). CO_2 production was calculated from the stoichiometry of the reactions. Byproduct and substrate concentrations used are given in Table 2.
[e] Data are from Tong et al., 1991 (*11*).
[f] Only 10 g/L yeast extract present.
[g] N.D.: Not detected.

Table 2
Substrate Consumption and Byproducts Formation in Cofermentations and Control Fermentations Shown in Table 1[a]

	Consumed substrates, g/L			Byproduct concentration, g/L					
Run #	Glycerol	Xylose	Glucose	D-Lactate	L-Lactate	Acetate	Succinate	Formate	Ethanol
1	2.14		2.05	1.48	0.08	1.10	0.16	1.11	0.39
2	2.38	1.17		0.91	0.08	1.08	0.33	1.12	0.57
3[b]	1.76			0.61	0.06	0.74	0.30	0.96	0.32
4			2.29	0.87	0.09	0.97	0.34	1.12	0.31
5		1.95		0.47	0.09	1.10	0.23	1.22	0.34
6				N.D.[c]	0.06	0.63	0.35	0.70	0.08

[a] All fermentations contain 10 g/L yeast extract and were incubated at 37°C for 30 h.
[b] Data are from Tong et al., 1991 (*11*).
[c] N.D.: Not detected.

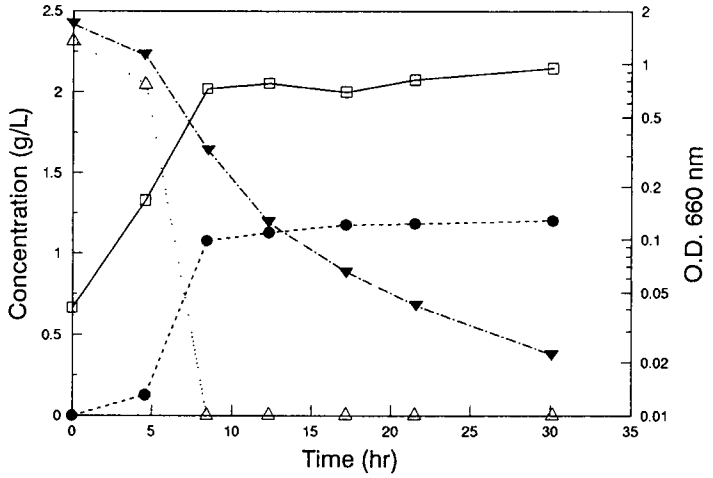

Fig. 1. Growth of *Escherichia coli* AG1/pTC1 and kinetics of 1,3-propanediol production and substrates consumption in the cofermentation of glycerol and glucose. —□— OD 660 nm; ---●--- 1,3-propanediol; ····△···· glycerol; —▼— glucose.

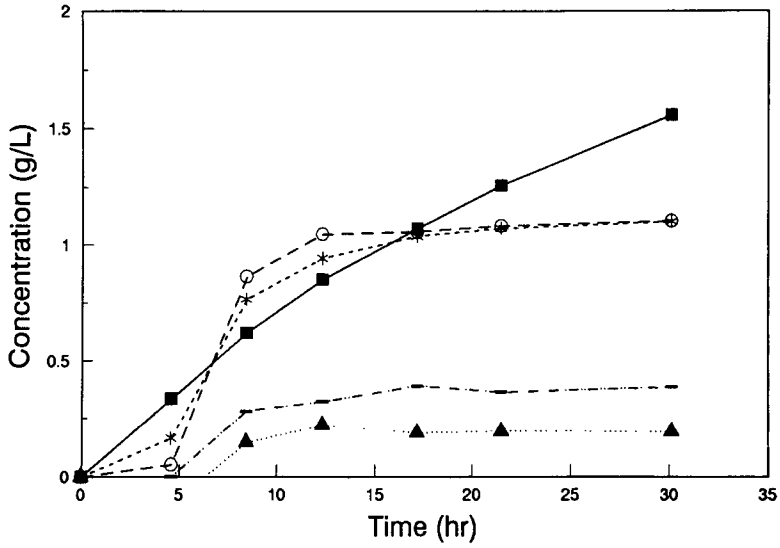

Fig. 2. Kinetics of byproduct formation of *Escherichia coli* AG1/pTC1 in the cofermentation of glycerol and glucose. —■— lactate, ---*---- acetate; ····▲···· succinate; --○-- formate; —··▬▬··— ethanol.

D-Lactate was the dominant form of lactate in all fermentations (except in the yeast extract control where no D-lactate was detected). The D-lactate was most likely derived via the methylglyoxal bypass, which is constitutive in *E. coli* (*13*).

The time-course of the glycerol/glucose cofermentation (Run 1) is shown in Figs. 1 and 2. Exponential cell growth ended at approx 8 h and appeared to correspond to the depletion of glycerol. However, the control fermentations without glycerol showed a similar leveling off of growth

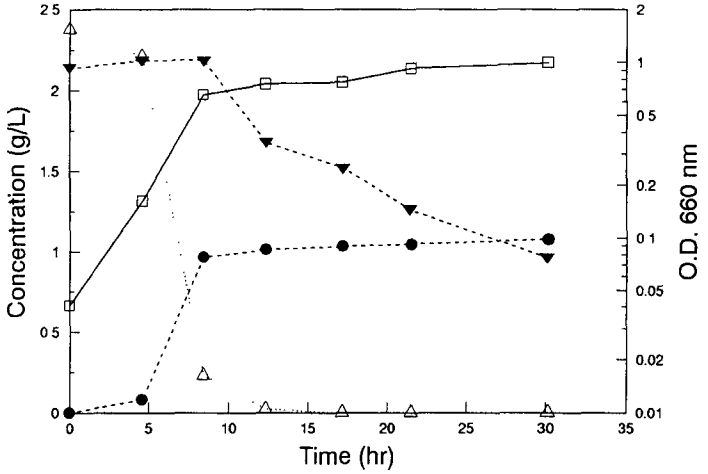

Fig. 3. Growth of *Escherichia coli* AG1/pTC1 and kinetics of 1,3-propanediol production and substrates consumption in the cofermentation of glycerol and xylose. —□— OD 660 nm; ---●--- 1,3-propanediol; ····△···· glycerol; ---▼--- xylose.

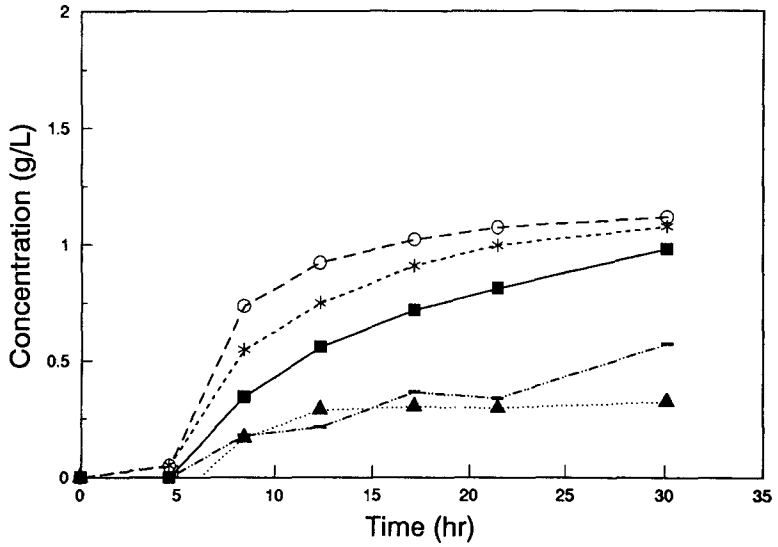

Fig. 4. Kinetics of byproduct formation of *Escherichia coli* AG1/pTC1 in the cofermentation of glycerol and xylose. —■— lactate; ---*--- acetate; ···▲··· succinate; --○-- formate; —··▬▬··— ethanol.

after approx 8 h (kinetic data for controls not shown), so it is unlikely that glycerol was growth limiting. 1,3-PD production followed the consumption of glycerol and leveled off when glycerol was depleted. Acetate, succinate, formate, and ethanol increased slightly after glycerol was depleted. Glucose was consumed and lactate produced throughout the fermentation.

The time-course of the glycerol/xylose cofermentation (Run 2) is shown in Figs. 3 and 4. As in Run 1, exponential growth ended after

Table 3
Maximum Theoretical Yields of 1,3-Propanediol from Glycerol

Overall mass balance

A. 8 Glycerol → 7 1,3-PD + 2 CO_2 + 4 H_2O
Yield = 0.875 mol/mol

Balance subject to topological constraints owing to the metabolic network

B. 4 Glycerol → 3 1,3-PD + acetate + CO_2 + 2 H_2O
Yield = 0.75 mol/mol

C. 3 Glycerol → 2 1,3-PD + acetate + formate + H_2O
Yield = 0.667 mol/mol

Balance with additional source of reducing equivalents

D. Glycerol + 2 [H] → 1,3-PD + H_2O
Yield = 1.0 mol/mol

E. 2 Glycerol + glucose → 2 1,3-PD + 2 acetate + 2 formate
Yield = 1.0 mol/mol

F. 5 Glycerol + 3 xylose → 5 1,3-PD + 5 acetate + 5 formate
Yield = 1.0 mol/mol

approx 8 h. 1,3-PD production followed glycerol utilization. There was an 8-h lag in xylose utilization, but once utilization started, the rate was similar to that of glucose. The byproducts appear to be derived from both substrates (with some contribution from the yeast extract).

DISCUSSION

Two important considerations for the development of fermentation processes for the production of chemicals are constraints owing to the mass balance (which include the electron balance) and constraints owing to kinetics (which include regulation). An overall mass balance gives the maximum theoretical yield of 1,3-PD from glycerol as 0.875 mol/mol (Table 3, reaction A). The theoretical yield is further constrained by the structure (or topology) of the metabolic network. No known microorganism is able to ferment glycerol entirely to 1,3-PD and CO_2. Instead, some other byproduct is produced, such as acetate (reducing the theoretical yield to 0.75 mol/mol [reaction B]), or more realistically, a mixture of byproducts, such as acetate and formate, reducing the theoretical yield to 0.667 mol/mol (reaction C).

It is possible to relieve the mass balance constraint by providing an exogenous source of reducing power. With the availability of extra reducing power, the maximum theoretical yield of 1,3-PD from glycerol calculated from the overall mass balance is 1.0 mol/mol (Table 3, reaction D). One way to provide reducing power to *E. coli* is in the form of sugar, i.e., by cofermentation. The fermentation of the sugar provides reducing power needed for the conversion of glycerol to 1,3-PD, giving a theoretical yield of 1.0 mol/mol (reactions E and F).

In theory, cofermentation is an effective way to improve the yield of 1,3-PD from glycerol. However, optimal production of 1,3-PD by cofermentation requires that:

1. Sugar (e.g., glucose or xylose) does not repress or interfere with the conversion of glycerol to 1,3-PD;
2. The 1,3-PD oxidoreductase is able to access NADH generated by sugar metabolism;
3. Glycerol is not diverted to products other than 1,3-PD (the yield will be reduced if some glycerol is converted to cell mass or to metabolic byproducts); and
4. Sugar is used as efficiently as possible (only enough sugar is used to provide the needed reducing power for 1,3-PD production and cell growth).

As shown in this research, with *E. coli* AG1/pTC1, the sugars and glycerol are used simultaneously (but to a greater extent for glucose than xylose). Although cofermentation of glycerol and glucose has been reported in some naturally occurring microorganisms (*14*), it has not been previously realized in *E. coli*. Two properties are required for the engineered *E. coli* to coferment glycerol and sugar. The first is that glycerol utilization must not be subject to catabolite repression. This is the case in wild-type *E. coli*, where the natural regulatory enzyme for glycerol dissimilation is glycerol kinase, the first enzyme after uptake (*4,15*). The second is that the presence of sugars does not repress or inhibit the *K. pneumoniae dha* pathway in *E. coli*.

The yield of 1,3-PD from glycerol during cofermentation is improved over that from glycerol alone. This improvement confirms the hypothesis that the availability of reducing power is one of the factors limiting the yield of 1,3-PD from glycerol. It also shows that the presence of sugars does not inhibit the pathway. However, the yield is not the theoretical maximum of 1.0 mol/mol, but rather 0.63 mol/mol for the glucose cofermentation and 0.55 mol/mol for the xylose cofermentation. This could be because NADH from sugar metabolism is not fully accessible to the 1,3-PD oxidoreductase responsible for 1,3-PD production or that glycerol is diverted into other products, such as cell mass or fermentation byproducts. Both possibilities are important considerations for metabolic pathway engineering.

Channeling and direct metabolite transfer via enzyme–enzyme complexes are known to occur in some metabolic pathways (*16*). Direct metabolite transfer in sugar metabolism could make the NADH (or some fraction thereof) unavailable. Furthermore, in addition to the 1,3-PD oxidoreductase gene, pTC1 also contains the glycerol dehydrogenase gene of the *K. pneumoniae dha* regulon. Any direct metabolite transfer of NADH between these two enzymes would reduce access of NADH from sugar metabolism to 1,3-PD oxidoreductase. Further work is needed to assess the role of direct metabolite transfer in this system.

The presence of the glycerol dehydrogenase and the DHA kinase genes on pTC1; gives the engineered *E. coli* a means to divert glycerol from 1,3-PD to DHAP. DHAP can be used for the production of cell mass or converted to metabolic byproducts, including D-lactate, formate, or acetate. This diversion could be prevented by eliminating glycerol dehydrogenase or DHA kinase activity. However, since the product of the glycerol dehydrogenase, DHA, is the inducer of the *dha* regulon, the elimination of DHA kinase, rather than glycerol dehydrogenase, seems to be preferable for improving the yield of 1,3-PD from glycerol during cofermentation.

In the optimal cofermentation system, the consumption of sugars should be optimized to reduce the cost of 1,3-PD production. Sugar is less expensive than glycerol, and should be used only for the production of cell mass and the generation of reducing power. On the other hand, it is theoretically possible to engineer an organism that could convert sugars directly to 1,3-PD, eliminating the need for glycerol altogether. In *E. coli* AG1/pTC1, the sugar consumption at the point where all glycerol is consumed is 0.18 mol glucose/mol glycerol and 0.16 mol xylose/mol glycerol. If the DHA kinase activity were eliminated as discussed in the previous paragraph, the consumption of sugars would be expected to increase, since sugar metabolism would then be the only possible source of reducing power and carbon for cell growth. In order to use sugars most efficiently for reducing power and cell growth, the preferred products from sugars are acetate and formate (or acetate, CO_2, and H_2). This is because production of acetate and formate provide a greater amount of NADH than the production of the other byproducts of *E. coli*—lactate, ethanol, and succinate.

The above results and discussion show that both the pathways involved in the direct synthesis of the product and the biochemical background of the host cell are important in metabolic pathway engineering. Both metabolite flux and cofactor regeneration must be carefully considered. Overexpressing the genes of the production pathway is not always enough. How to optimize the interaction of a novel pathway within the host cell and how to modify topological and kinetic constraints associated with the metabolic system are also of importance.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation, BCS-8910077, and the Graduate School of the University of Wisconsin.

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