Utilizing Succinic Acid as a Glucose Adjunct in Fed-Batch Fermentation: Is Butane a Feedstock Option in Microbe-Catalyzed Synthesis?

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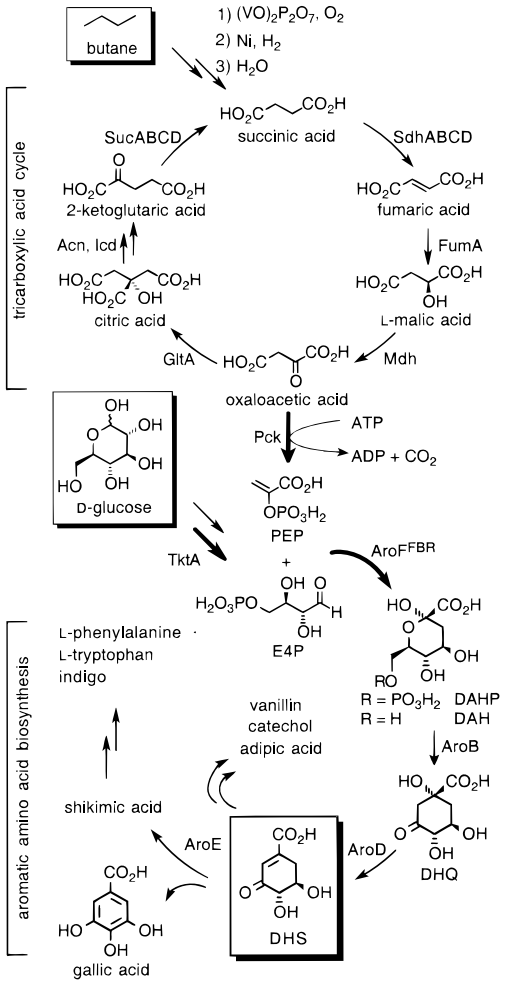
Although the carbohydrate phosphotransferase system[1] supports rapid bacterial growth, this transport mechanism imposes an upper limit on the yields and concentrations of chemicals which can be synthesized when microbes such as *Escherichia coli* employ glucose as a carbon source. Phosphotransferase-catalyzed phosphoryl group transfer from phosphoenolpyruvic acid (PEP) simultaneously drives glucose transport and glucose phosphorylation. Because byproduct pyruvic acid is not significantly recycled to PEP in phosphotransferase-utilizing microbes, three carbon atoms are lost from chemical synthesis for every six carbon atoms transported into the cytoplasm.[2] A possible means for circumventing this limitation has been evaluated which exploits butane-derived[3] succinic acid (Scheme 1) as an adjunct to starch-derived glucose. By overexpressing selected enzymes and evading catabolite repression, succinic acid is apparently exploited as a transportable precursor of PEP, resulting in the synthesis of increased concentrations of 3-dehydroshikimic acid (DHS) by *E. coli*. DHS is a potent antioxidant[4] and the most advanced common biosynthetic intermediate in the biocatalytic synthesis of adipic acid,[5] catechol,[6] vanillin,[7] shikimic acid,[8] phenylalanine,[9] tryptophan,[10] and indigo.[11]

The native tricarboxylic acid cycle was used to convert transported glucose adjuncts into oxaloacetic acid (Scheme 1). Plasmid-localized, *pck*-encoded[12] PEP carboxykinase then converted oxaloacetic acid into PEP. This increased level of PEP generation was channeled into the common pathway of aromatic amino acid biosynthesis (Scheme 1) by plasmid-localized, P_{tac}*aroF*^{FBR}-encoded[13] 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase. Because D-erythrose 4-phosphate (E4P) availability can limit DAHP synthase activity, the impact of plasmid-localized *tktA*-encoded[14] transketolase was examined.

Scheme 1



Fed-batch fermentor cultivation of DHS-synthesizing constructs relied on a dissolved O_2 sensor to control addition of the adjunct/glucose mixtures.[15] This addition strategy prevents glucose concentrations from reaching a level[16] where catabolic repression of adjunct transport[17] occurs.

DHS (57 g/L) concentrations synthesized (Table 1) from succinate/glucose (1:1, mol/mol) by KL3/pKL6.218A, which overexpressed DAHP synthase, transketolase, and PEP carboxykinase, were significantly higher than DHS (44 g/L) concentrations synthesized from glucose. Another measure of the increased carbon flow directed into aromatic amino acid biosynthesis is the accumulation (Figure 1, Table 2) of 3-deoxy-D-*arabino*-heptulosonic acid (DAH), 3-dehydroquinic acid (DHQ), and gallic acid. DAH results from enzymatic hydrolysis of DAHP, while gallic acid is derived from DHS.[18] Significantly higher concentrations of DHQ were synthesized from succinate/glucose versus glucose by KL3/pKL6.218A (Table 2). While no DAH accumulation was observed (Table 2) during cultivation of KL3/pKL6.218A on glucose, substantial DAH was produced by this construct from the succinate/glucose mixture.

The importance of PEP carboxykinase is evident (Table 1) from the reduced concentrations of DHS (46 g/L) synthesized from

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Table 1. Products Synthesized by KL3 as a Function of Plasmid and Carbon Source

	pKL4.79B	pKL4.12A	pKL6.198A	pKL6.218A
tktA	−	+	−	+
pck	−	−	+	+
		Glucose		
[DHS], g/L	36	46	nd[f]	44
DHS yield, %[a]	22	28	nd	28
total yield, %[b]	26	33	nd	32
units activity[c]	0.04	0.04	nd	0.48
		Glucose/Succinate		
[DHS], g/L	nd	46	34	57
DHS yield, %[d]	nd	24	21	29
total yield, %[e]	nd	29	25	37
units activity[c]	nd	0.04	0.77	044

[a] $mol_{DHS}/mol_{glucose}$. [b] $mol_{DHS+DAH+DHQ+gallate}/mol_{glucose}$. [c] PEP carboxykinase, μmol/min/mg. [d] $mol_{DHS}/mol_{glucose+succinate}$. [e] $mol_{DHS+DAH+DHQ+gallate}/mol_{glucose+succinate}$. [f] Not determined.

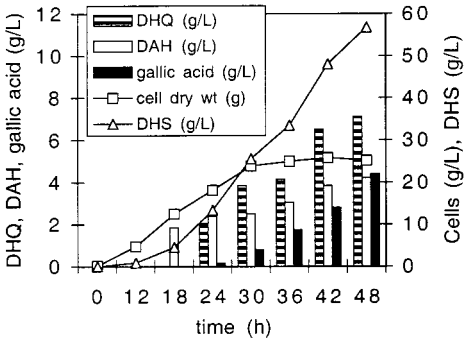

Figure 1. Cultivation of KL3/pKL6.218 on succinate/glucose.

Table 2. Products Synthesized by KL3/pKL6.218A as a Function of Glucose Adjunct

	glucose	glucose/ succinate	glucose/ malate	glucose/ 2-ketoglutarate
[DHS], g/L	44	57	62	58
DHS yield, %[a]	28	29	30	29
[DAH], g/L	0	4.2	5.4	4.8
[DHQ], g/L	4.5	7.1	9.1	7.9
[gallate], g/L	3.8	4.4	4.6	4.3
total yield, %[b]	32	37	38	37

[a] $mol_{DHS}/mol_{glucose+adjunct}$. [b] $mol_{DHS+DAH+DHQ+gallate}/mol_{glucose+adjunct}$.

succinate/glucose by KL3/pKL4.124A, which differs from KL3/pKL6.218A only in the absence of PEP carboxykinase overexpression. DHS concentrations synthesized by KL3/pKL4.124A were essentially the same (Table 1), irrespective of whether glucose or succinate/glucose were the source of carbon. A precipitous drop in synthesized DHS was observed (Table 1) for KL3/pKL6.198A, which overexpresses PEP carboxykinase but not transketolase. The 34 g/L of DHS synthesized by KL3/pKL6.198A from the succinate/glucose mixture is comparable to the 36 g/L of DHS synthesized from glucose by KL3/pKL4.79B, which lacks overexpressed PEP carboxykinase and transketolase. The decline in DHS synthesized by KL3/pKL6.198A is consistent with E4P availability limiting DAHP synthase activity.[2a,14]

Other tricarboxylic acid cycle intermediates that successfully served as glucose adjuncts included L-malic acid and 2-ketoglutaric acid. DHS, DAH, DHQ, and gallic acid concentrations synthesized by KL3/pKL6.218A from mixtures (1:1, mol/mol) of L-malate/glucose and 2-ketoglutarate/glucose were similar (Table 2) to the concentrations of these metabolites synthesized from succinate/glucose. No increase in DHS concentration was observed when citric acid was used as a glucose adjunct. This is consistent with the inability of *E. coli* to transport citric acid.[19] Fumaric acid was not sufficiently soluble in water to generate the highly concentrated carbon source solutions required to avoid large increases in culture volume during fermentor runs.

Titer improvements have been reported when transketolase is overexpressed along with PEP synthetase, an enzyme which catalyzes recycling of phosphotransferase-produced pyruvic acid to PEP.[2a] However, constructs overexpressing PEP synthetase display problematic growth characteristics under fermentor conditions.[2a] Improvements in product titer have also been reported in *E. coli* overexpressing transketolase when the phosphotransferase system is mutationally inactivated and glucose is transported via an upregulated galactose permease.[20] The growth characteristics of these constructs have not yet been reported under fermentor conditions. Titer improvements under both shake flask[2b] and fermentor conditions[21] have been observed when D-xylose and L-arabinose were used as carbon sources as a consequence of ATP-driven permease transport of D-xylose and L-arabinose.[19] Unfortunately, pentose streams sufficiently pure for microbial fermentation are not commercially available.

In relation to the aforementioned strategies for improving product titers in microbe-catalyzed synthesis, use of succinic acid as a glucose adjunct is distinguished by the combination of genetic manipulation and carbon source modification. Under fermentor conditions, normal growth rates of the *E. coli* constructs are observed (Figure 1), and carbon flow into aromatic amino acid biosynthesis is increased as reflected by DHS titers as well as by DAH and DHQ titers (Table 2). Pure succinic acid is also commercially available.[3] Although current pricing of succinic acid restricts its use to microbe-catalyzed synthesis of higher value-added chemicals, economies of scale remain to be fully exploited in succinic acid manufacture. In light of the abundant availability of butane from liquefiable petroleum gases,[22] use of butane-derived succinic acid as a glucose adjunct demonstrates how a fossil fuel feedstock can be combined with a renewable feedstock to create a carbon source for microbe-catalyzed chemical synthesis which is superior to glucose.

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Supporting Information Available: Fermentation and assay procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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